



# Evolution of Bivalvia: Multi-level phylogenetic and phylogenomic reconstructions within Bivalvia (Mollusca) with emphasis on resolving familial relationships within Archiheterodonta (Bivalvia: Heterodonta).

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Evolution of Bivalvia: Multi-level phylogenetic and phylogenomic reconstructions within  
Bivalvia (Mollusca) with emphasis on resolving familial relationships within  
Archiheterodonta (Bivalvia: Heterodonta).

A dissertation presented  
by  
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to

The Department of Organismic and Evolutionary Biology

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**Evolution of Bivalvia: Multi-level phylogenetic and phylogenomic reconstructions within Bivalvia (Mollusca) with emphasis on resolving familial relationships within Archiheterodonta (Bivalvia: Heterodonta)**

Abstract

With an estimated 8,000-20,000 species, bivalves represent the second largest living class of molluscs (Bieler *et al.* 2013). Revived interest in molluscan phylogeny has resulted in a torrent of molecular sequence data from phylogenetic, mitogenomic, and phylogenomic studies. Despite recent progress, basal relationships of the class Bivalvia remain contentious, owing to conflicting hypotheses often between morphology and molecules.

In Chapter 1, the utility of four nuclear protein-encoding genes—ATP synthase  $\beta$ , elongation factor-1 $\alpha$ , myosin heavy chain type II, and RNA polymerase II— were evaluated for their adequacy in resolving the basal relationships within Bivalvia. Marked insensitivity of the basal tree topology to dataset manipulation was indicative of signal robustness in these four genes, but resolution was missing in some key areas. Subsequently, a phylogenomic study using transcriptomic data was designed to re-evaluate the bivalve Tree of Life.

In Chapter 2, I provide the first phylogenomic analysis of Bivalvia in an attempt to resolve deep divergences within this group. All six major lineages of bivalves (Archiheterodonta, Anomalodesmata, Inaequidonta, Palaeoheterodonta, Protobranchia, and Pteriomorphia) were sampled resulting in 31 newly sequenced Illumina-based



bivalve transcriptomes. This constitutes the most comprehensive phylogenomic dataset to date for inferring deep relationships within Bivalvia. Subsequent analyses obtained robust resolution of major bivalve lineages, largely corroborating classical taxonomic relationships based mostly on paleontological and morphological data.

In Chapter 3, a multi-locus phylogeny of archiheterodont bivalves was constructed to resolve relationships among constituent families; the relationships and internal phylogeny of Archiheterodonta remain poorly understood. By virtue of the contentious placement within Bivalvia and lack of internal phylogenetic work, Archiheterodonta (including Astartidae, Carditidae, Crassatellidae, and Condyllocardiidae) remain an enigmatic clade of phylogenetic interest. Here I investigate relationships within Archiheterodonta using molecular information from six genes (28S rRNA, 18S rRNA, histone H3, cytochrome *c* oxidase subunit I, internal transcribed spacer 2, and cytochrome *b*), including the first molecular sequence information for Condyllocardiidae.

Lastly, in Chapter 4, I investigated the potential cryptic speciation within a complex of carditid bivalves from the Gulf of California (Archiheterodonta, Carditidae). *Carditamera bajaensis*, new species, is described from semi-infaunal specimens collected in the intertidal zone in the Gulf of California, Baja California Sur, Mexico. This species, overlapping in distribution with the congeneric *C. affinis*, yet subsequently found to be genetically distinct, was differentiated initially due to life mode and shell morphology.

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## **DEDICATION**

This dissertation is dedicated to my grandparents.

Esta tesis está dedicada a mis abuelos.

## INTRODUCTION

The molluscan class Bivalvia consists of mostly laterally compressed animals with a shell made of calcium carbonate, which are enclosed in a bilaterally symmetrical, dorsally hinged shell composed of two distinct valves held together by a single or a pair of adductor muscles (Giribet & Distel 2003). To date, there are approximately 8,000 to 20,000 extant described bivalve species, characterized by the presence of a foot for locomotion and siphons used in feeding (Bieler *et al.* 2013). This ancient lineage of molluscs is both economically important as source of animal protein and ecologically important, where large assemblages of suspension feeding bivalves can be major contributors to ecosystem function (Newell 2004; Drumbauld *et al.* 2009).

Taxonomic classifications of Bivalvia have been subject to many revisions and reorganizations owing to the discordant phylogenetic signal from previously analyzed morphological, paleontological and molecular datasets. Morphologically, bivalves have been placed in higher-level taxonomic groups based on single character systems such as hinge dentition patterns, structure of ctenidia (used in respiration and feeding), and stomach organization, or complex characteristics of external shell morphology, shell ultrastructure and paleontological data; yet no consensus has been reached on a classification due to marked discordance between taxonomic hypotheses. The lack of stability in taxonomic classifications of bivalves has impeded the identification of higher-level bivalve lineages. Nonetheless, within the class many classically recognized clades - Pteriomorphia, Protobranchia, Palaeoheterodonta, Heterodonta and Anomalodesmata - have been used to describe members of the Bivalvia, though relationships among these groups have yet to be further tested (Bieler *et al.* in press).

Protobranchia is a group of exclusively marine bivalves having small thin shells that are characterized by varying degrees of hinge dentition and simple ctenidial structure (Giribet & Distel 2003). Pteriomorpha are found in both freshwater and marine habitats, and includes, among other groups, the oysters and mussels (Giribet & Wheeler 2002). Morphologically, they are characterized by relatively large, thin, compressed shells, which can be smooth or ribbed and are predominantly anchored to substrates using byssal threads or valve cementation (Bieler & Mikkelsen 2006). Palaeoheterodonta are both marine and limnic, with larger, often grooved shells that can be ribbed or smooth and the group includes the freshwater mussels and pearl mussels (Giribet and Wheeler 2002). Heterodonta includes groups such as clams, cockles, and comprises two clades: Archiheterodonta, whose members share the presence of a unique hemoglobin, and Euheterodonta. Inaequidonta has been recently recognized as a clade based on molecular data and constitutes a diverse subclade of Euheterodonta, found both in marine and limnic environments, and including the widest range of shell sizes among all bivalve groups. Euheterodonts can have smooth or sculptured valves and have well developed siphons (Giribet & Distel 2003; Taylor *et al.* 2007). Lastly, comprising Euheterodonta along with Inaequidonta, Anomalodesmata, was formerly treated as its own class (e.g., Newell 1965) having a highly variable shell shape and size, yet united by reduced hinge dentition and the clade includes the carnivorous bivalves (Bieler & Mikkelsen, 2006; Harper *et al.* 2006).

Bivalves first appear in the fossil record in the lower Cambrian and along with gastropods, became the dominant benthic fauna through the Mesozoic and Cenozoic after the Permian mass extinction, prior to which the fossil record is dominated by brachiopod

taxa (Fraiser & Bottjer 2007). Of the six lineages, Protobranchia includes the earliest bivalves found in the lower Cambrian, whereas fossil Palaeoheterodonta are found in the Late Paleozoic but dominate in the Mesozoic. The earliest fossil Heterodonta (Archiheterodonta + Euheterodonta) are found in the Paleozoic (Anderson 1998). During the Paleozoic, retention of the byssus and cementation allowed for the colonization of hard substrates and harsher environments, concurrently the Pteriomorphia and Archiheterodonta diversified (Giribet 2008). Subsequent lineages diversified throughout the Paleozoic, though were diminished following the Permo-Triassic mass extinction (Slack-Smith 1998). The diverse and relatively complete fossil record provides thus beneficial paleobiological information to reconstruct divergence times for ancient rapid radiations in this group.

Early molecular phylogenetic analyses had failed to recover the monophyly of Bivalvia. However, latter studies employing next-generation sequencing (NGS) techniques including phylogenomic reconstructions with large molecular datasets recover the monophyly of bivalves and place bivalves as the sister group to gastropods (Kocot *et al.* 2011) or to a gastropod + scaphopod clade (Smith *et al.* 2011), but conflicts in morphological and molecular datasets persist within major molluscan lineages, particularly Bivalvia. The mutual monophyly of Protobranchia and Autobranchia (Pteriomorphia + Paleoheterodonta + Archiheterodonta + Euheterodonta) is recovered, yet key taxa imperative to the phylogenetic structure within Bivalvia were not sampled, as the aim of these studies was not to resolve the internal relationships of each molluscan class (Kocot *et al.* 2011; Smith *et al.* 2011). Moreover, mitogenomic analyses using full mitochondrial genomes recovered the diphyly of Bivalvia, by virtue of *Solemya velum*



nesting within Gastropoda (Plazzi *et al.* 2013), suggesting that a mitogenomic framework was unable to resolve relationship within Bivalvia or to delineate basal molluscan relationships among the eight classes (Plazzi *et al.* 2013). Incongruence among mitogenomic, mitochondrial and nuclear gene datasets has been attributed to multiple characteristics within the mitochondrial genome; such discrepancies include degrees of recombination, modes of inheritance, incidence of introns, and in proportion of mitochondrial genome to haploid nuclear genome (Sharma *et al.* 2012).

Despite recent general congruence of the phylogenetic placement of major bivalve lineages, support for several critical basal nodes is lacking, especially in light of recent molecular data analyses using mitochondrial (e.g., Plazzi and Passamonti 2010; Plazzi *et al.* 2011, 2013) versus nuclear (e.g., Sharma *et al.* 2012) genes. Particularly two major clades of the “backbone” of bivalve evolution have yet to converge on robust phylogenetic placement: Protobranchia and Archiheterodonta (Bieler *et al.* 2013). Recent molecular investigations have recovered the once contentious monophyly of Protobranchia (Kocot *et al.* 2012; Smith *et al.* 2011; Sharma *et al.* 2012, 2013). Relationships within Protobranchia have not yet stabilized and every iteration of relationships between the three superfamilies: Solemyida, Nuculida, and Nuculanida have been proposed based on morphological information, paleontological data or have been recovered based on molecular sequence information (e.g., Opponobranchia [Nuculida + Solemyida] and a clade of Nuculanida + Autobranchia) (Giribet & Wheeler 2002; Giribet & Distel 2003; Giribet 2008; Wilson *et al.* 2010). As of late, reconstructions of internal protobranch phylogeny favor the clade (Nuculida + Nuculanida) as the sister group to Solemyida (Sharma *et al.* 2013). In addition to molecular sequence information, several

morphological synapomorphies unite this clade, including: “primitive” eponymous protobranch gill; the palp proboscides (absent in the solemyoids, likely a consequence of obligate chemosymbiosis, as with reductions of the alimentary system); and characteristic taxodont dentition (Coan *et al.* 2000).

Likewise, recent reconstructions of relationships of Bivalvia have yet to converge on the phylogenetic placement of Archiheterodonta (Giribet & Wheeler, 2002; Wilson *et al.* 2010; Plazzi & Passamonti 2010; Carter *et al.* 2011; Plazzi *et al.* 2011; Sharma *et al.* 2012). Phylogenetic evidence for a sister group relationship between Archiheterodonta and Euheterodonta (Anomalodesmata + Inaequidonta) is prevalent (e.g., Campbell 2000; Park & Ó Foighil 2000; Giribet & Wheeler 2002; Dryer *et al.* 2003; Giribet & Distel 2003; Taylor & Glover 2006; Harper *et al.* 2006; Taylor *et al.* 2007). Archiheterodonta has been recovered in the traditional placement as the sister group to the remaining Heterodonta (Giribet & Wheeler 2002; Carter *et al.* 2011), closely related to members of Pteriomorphia (Plazzi *et al.* 2011), or related to a derived group, Anomalodesmata (Plazzi & Passamonti 2010)—the latter relationships based solely on mitochondrial gene sequence information, recovering vastly different evolutionary histories for this group. Nevertheless, recent molecular and combined molecular and morphological phylogenies have begun to converge on the placement of Archiheterodonta, forming a clade with an ancient lineage of bivalves, Paleoheterodonta, which in turn constitutes the sister group to Euheterodonta (Wilson *et al.* 2010; Sharma *et al.* 2012; Bieler *et al.* in press).

This dissertation aims to contribute to the understanding of bivalve systematics and phylogeny using both phylogenetic and phylogenomic approaches; identify stable taxonomic groups within this lineage; test the position of Archiheterodonta using a

phylogenomic framework, and investigate the internal phylogeny and species delimitations within Archiheterodonta.

In Chapter 1, the utility of four nuclear protein-encoding genes—ATP synthase  $\beta$ , elongation factor-1 $\alpha$ , myosin heavy chain type II, and RNA polymerase II— are evaluated for resolving the basal relationships of Bivalvia. Marked incongruence of phylogenetic signal in datasets heavily represented by nuclear ribosomal genes versus mitochondrial genes has also impeded consensus on the type of molecular data best suited for investigating bivalve relationships. Despite the stability of the basal tree topology to dataset manipulation, indicative of signal robustness in these four genes, support for several imperative basal nodes was lacking. To combat this, in Chapter 2, I employed a phylogenomic approach to help resolve relationships of Bivalvia. All five major lineages of bivalves (Archiheterodonta, Euheterodonta [including Anomalodesmata], Palaeoheterodonta, Protobranchia and Pteriomorphia) were sampled, resulting in 31 newly sequenced bivalve transcriptomes and subsequent analyses obtained robust and stable resolution of bivalve lineages. Analyses of resultant supermatrices constitute the most comprehensive phylogenomic dataset to date for any animal group.

In Chapter 3, I undertake the phylogenetic reconstruction of Archiheterodonta using a multi-locus dataset to resolve relationships among constituent families, one of the remaining bivalve clades whose internal phylogeny has not been resolved. This is in striking contrast to commensurate undertakings of taxonomic revisions throughout Bivalvia, where phylogenetic reconstructions have been published focusing on internal relationships of Protobranchia (Sharma *et al.* 2012), Pteriomorphia (Healy *et al.* 2000, Temkin 2006), Palaeoheterodonta (Graf & Cummings 2006) and for members of the

Euheterodonta (Harper *et al.* 2006, Mikkelsen *et al.* 2006, Taylor *et al.* 2007).

Archiheterodonta contains some of the oldest known bivalve fossils, dating back to the Silurian or even the Ordovician and is comprised of four families: Astartidae, Carditidae, Crassatellidae, and Condyllocardiidae. These bivalves are exclusively marine, predominantly infaunal (though some byssate forms occur), suspension feeders that lack siphons. Astartidae includes 11 living genera (ca. 50 species) that inhabit arctic to temperate waters. Crassatellidae includes 9 living genera (ca. 40 species) and have a global distribution, though most are found predominantly in tropical and subtropical regions. Carditidae includes 16 living genera (ca. 50 species), distributed globally with the exception of the polar regions. Condyllocardiidae includes 21 living genera (ca. 65 species) distributed globally, though little is known about the biology of these species. Here I investigate relationships within Archiheterodonta using molecular information from six genes (28S rRNA, 18S rRNA, histone H3, cytochrome *c* oxidase subunit I, internal transcribed spacer 2, and cytochrome *b*) to assess the phylogenetic placement and validity of constituent families. All four families were sampled, including the first molecular sequence information for Condyllocardiidae.

Lastly in Chapter 4, I identified cryptic speciation in a Carditid bivalve from the Gulf of California (Mollusca, Bivalvia, Archiheterodonta, Carditidae). Where, *Carditamera bajaensis*, a new species, is subsequently described from semi-infaunal specimens collected in the intertidal zone in the Gulf of California, Baja California Sur, Mexico. The new species resembles *Carditamera affinis* (G. B. Sowerby I, 1833), the only valid *Carditamera* species known from within the Gulf of California, with which it has been mistaken, but it differs in shell structure and most conspicuously in life mode –

semi-infaunal for *C. bajaensis* versus byssally attached to hard substrata for *C. affinis*. I constructed haplotype networks from two mitochondrial genes (16S rRNA and cytochrome *b*) and one nuclear gene (internal transcribed spacer 2) which indicate a clear genetic break between *C. affinis* and *C. bajaensis*, as suspected initially due to their different modes of life and shell morphology. This pair of species, *C. affinis* and *C. bajaensis*, overlapping in distribution yet genetically distinct, possibly indicates ecological speciation.

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## CHAPTER 1

### **Phylogenetic analysis of four nuclear protein-encoding genes largely corroborates the traditional classification of Bivalvia (Mollusca).**

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## Abstract

Revived interest in molluscan phylogeny has resulted in a torrent of molecular sequence data from phylogenetic, mitogenomic, and phylogenomic studies. Despite recent progress, basal relationships of the class Bivalvia remain contentious, owing to conflicting morphological and molecular hypotheses. Marked incongruity of phylogenetic signal in datasets heavily represented by nuclear ribosomal genes versus mitochondrial genes has also impeded consensus on the type of molecular data best suited for investigating bivalve relationships. To arbitrate conflicting phylogenetic hypotheses, we evaluated the utility of four nuclear protein-encoding genes—ATP synthase b, elongation factor-1a, myosin heavy chain type II, and RNA polymerase II—for resolving the basal relationships of Bivalvia. We sampled all five major lineages of bivalves (Archiheterodonta, Euheterodonta [including Anomalodesmata], Palaeoheterodonta, Protobranchia, and Pteriomorphia) and inferred relationships using maximum likelihood and Bayesian approaches. To investigate the robustness of the phylogenetic signal embedded in the data, we implemented additional datasets wherein length variability and/or third codon positions were eliminated. Results obtained include (a) the clade (Nuculanida + Opponobranchia), i.e., the traditionally defined Protobranchia; (b) the monophyly of Pteriomorphia; (c) the clade (Archiheterodonta + Palaeoheterodonta); (d) the monophyly of the traditionally defined Euheterodonta (including Anomalodesmata); and (e) the monophyly of Heteroconchia, i.e., (Palaeoheterodonta + Archiheterodonta + Euheterodonta). The stability of the basal tree topology to dataset manipulation is indicative of signal robustness in these four genes. The inferred tree topology corresponds closely to those obtained by datasets dominated by nuclear ribosomal genes (18S rRNA and 28S rRNA), controverting recent taxonomic actions based solely upon mitochondrial gene

phylogenies.

Keywords: Protobranchia, Autobranchia, Heteroconchia, Phylogeny, Protein-encoding genes

## Introduction

Bivalvia is the second largest class of mollusks after Gastropoda and is comprised of aquatic (predominantly marine), bilaterally symmetrical animals characterized by a laterally compressed body enclosed in a bivalved shell and the lack of a radular apparatus. Extant bivalves are abundantly represented from intertidal to hadal marine environments and many species have significant commercial importance. Five distinct groups of bivalves are often recognized: the protobranchs (solemyoids, nukuloids, and nukulanooids); the pteriomorphians (mussels, scallops, oysters, and arks); the palaeoheterodonts (most freshwater mussels and trioniids); the archiheterodonts (which include members with hemoglobin); and the euheterodonts, including Anomalodesmata (the most speciesrich and widely distributed group of bivalves) (Bieler and Mikkelsen, 2006; Giribet, 2008).

The monophyly of Bivalvia is supported by numerous morphological apomorphies, but has historically proven elusive to demonstrate based on molecular sequence data, owing to early limitations in sampling of molecular loci and/or taxa (e.g., Steiner and Müller, 1996; Adamkewicz et al., 1997; Campbell et al., 1998; Giribet and Carranza, 1999; Giribet and Wheeler, 2002; Giribet and Distel, 2003; Giribet et al., 2006; but see Wilson et al., 2010). Recent application of second-generation sequencing techniques to outstanding questions of molluscan systematics has corroborated the monophyly of bivalves and their sister relationship to either Gastropoda (Kocot et al., 2011; based on 308 genes) or the clade (Gastropoda + Scaphopoda) (Smith et al., 2011; based on 1185 genes). Similarly, morphological cladistic analyses in concert with molecular phylogenies have elucidated relationships of many internal bivalve clades, such as within Pteriomorphia (Canapa et al., 2000; Matsumoto and Hayami, 2000; Steiner and Hammer, 2000; Matsumoto, 2003; Tëmkin,



2006, 2010; Waller, 2006), Unionida (Hoeh et al., 1999; Graf, 2000; Graf and Ó Foighil, 2000; Huff et al., 2004; Graf and Cummings, 2006), Anomalodesmata (Dreyer et al., 2003; Harper et al., 2006), and other Heterodonta (Canapa et al., 1999, 2001, 2003; Park Ó Foighil, 2000; Campbell et al., 2004; Williams et al., 2004; Taylor et al., 2005, 2007, 2009, 2011; Mikkelsen et al., 2006; Taylor and Glover, 2006).

Following decades of dispute concerning the position of major bivalve lineages (Fig. 1.1; reviewed by Giribet and Wheeler, 2002; Giribet, 2008), certain aspects of basal bivalve phylogeny have begun to stabilize. For example, although protobranch monophyly has long been contentious (Campbell et al., 1998; Giribet and Wheeler, 2002; Giribet and Distel, 2003; Wilson et al., 2010), the mutual monophyly of Protobranchia (Nuculanida + Opponobranchia sensu Giribet, 2008) and Autobranchia—the group comprised of all bivalves with ctenidia modified for filter-feeding—was obtained in a recent phylogenomic analysis sampling all three protobranch superfamilies (Smith et al., 2011; although Archiheterodonta and Anomalodesmata were not sampled). Similarly, the monophyly of Heteroconchia (Palaeoheterodonta + Archiheterodonta + Euheterodonta [including Anomalodesmata]) was obtained previously (Giribet and Wheeler, 2002; Wilson et al., 2010), with phylogenetic studies corroborating either a sister relationship of Archiheterodonta and Euheterodonta (Giribet and Wheeler, 2002; Taylor et al., 2007), or of Archiheterodonta and Palaeoheterodonta (Wilson et al., 2010). A noteworthy commonality of these phylogenetic studies is the use of datasets heavily or exclusively represented by nuclear ribosomal genes (18S rRNA and 28S rRNA).

A notable exception to this accruing consensus was a molecular phylogeny based on four genes (18S rRNA, 28S rRNA, histone H3, and cytochrome c oxidase subunit I) that

recovered a sister relationship of Nuculanida and Archiheterodonta, and a more basal divergence of Palaeoheterodonta (Giribet and Distel, 2003). Part of the discrepancy may have been attributable to the analytical treatment of the gene histone H3, which was newly sequenced for Bivalvia at the time. In that study, the optimal parameter set selected for parsimony analysis assigned all substitutions the same weight. Reexamination of histone H3 performance across various invertebrate taxa has demonstrated the suppression of phylogenetic signal of this gene given equal weighting of transversion and transition events—typically caused by the disproportionate influence of the nuclear ribosomal genes—and ensuing false topological incongruity (Sharma et al., 2011). Nevertheless, certain aspects of the topology of Giribet and Distel (2003) were corroborated by subsequent analyses (e.g., Wilson et al., 2010), specifically with respect to derived relationships and the monophyly of Euheterodonta (including Anomalodesmata).

In spite of these advances toward a stable bivalve phylogeny, basal relationships have once again come into question owing to topologies obtained using mitochondrial genes and genomes. One such study, examining doubly uniparental inheritance (DUI) of the mitochondrial genome (which has heretofore been documented in Mytilida, Unionida, and Venerida; reviewed by Breton et al., 2007), constructed a 12-mitochondrial gene phylogeny sampling only unionoids, veneroids, and three groups of pteriomorphians (Pectinida, Ostreida, and Mytilida) (Doucet-Beaupré et al., 2010). Although this sampling was deemed sufficient for the purposes of that study (i.e., mapping gains and losses of DUI in Autobranchia), the omission of several major groups could have engendered the unusual sister relationship of Pteriomorphia and Euheterodonta (not including Anomalodesmata) to

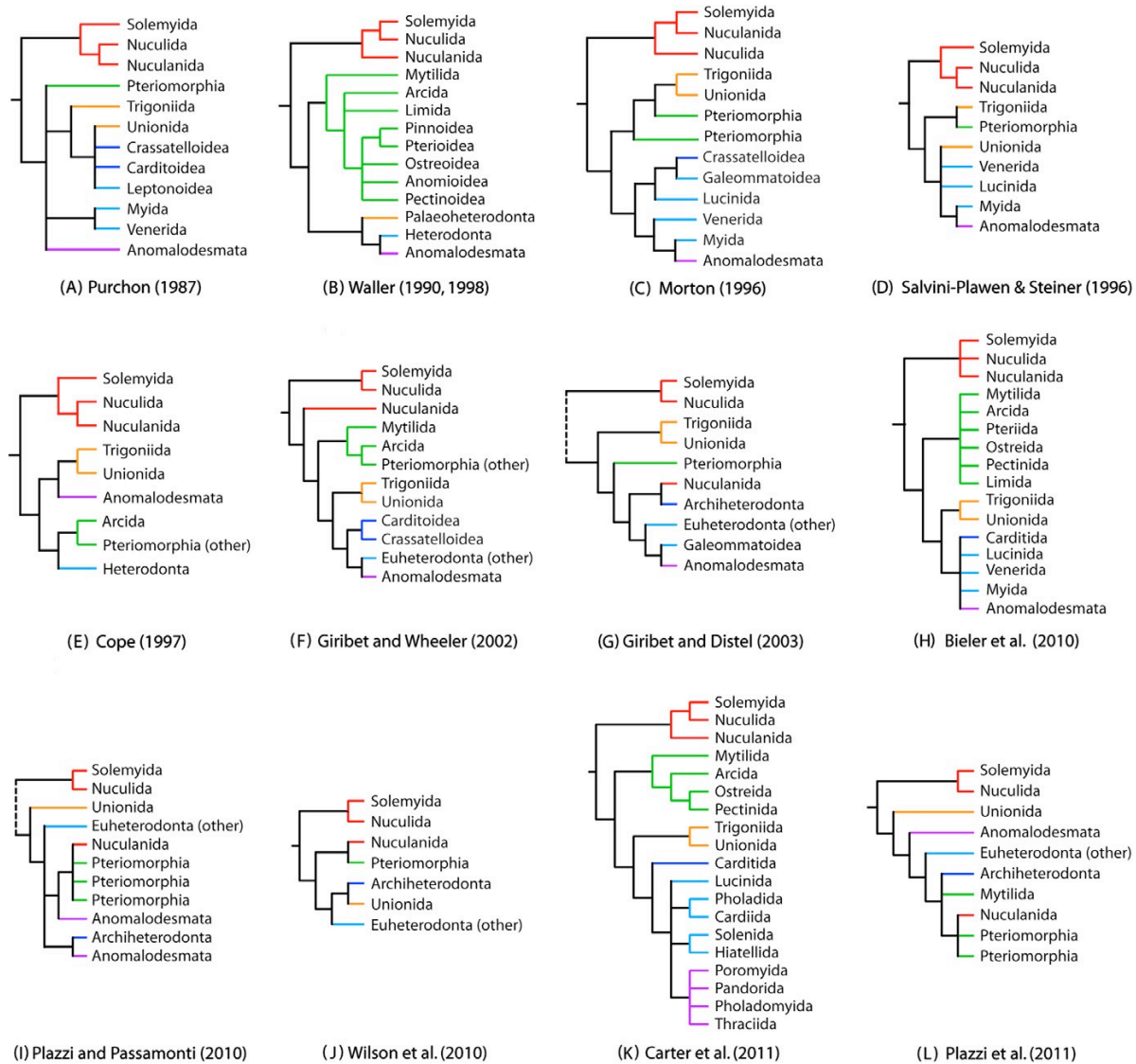
the exclusion of Palaeoheterodonta. Furthermore, two recent phylogenetic analyses, both based exclusively on four mitochondrial genes (12S rRNA, 16S rRNA, cytochrome *c* oxidase subunit I, and cytochrome *b*), recovered highly counterintuitive topologies. In the first case, bivalves were recovered as polyphyletic owing to the placement of Solemyida and Nuculida, and the remaining relationships obtained included polyphyly of Anomalodesmata, and Nuculanida clustering with Pteriomorphia (Fig. 1.1I; Plazzi and Passamonti, 2010). A subsequent rendition with increased taxonomic sampling obtained the monophyly of Bivalvia, but a markedly different topology (Fig. 1.1L; Plazzi et al., 2011). The authors proposed the name “Amarsipobranchia” for all bivalves except Nuculida, Solemyida, and Unionida, based upon a gill character that does not in fact occur in all Archiheterodonta, Euheterodonta, or Pteriomorphia—but “has most probably to be considered as a symplesiomorphy of this group” (Plazzi et al., 2011, p. e27147). The authors concluded that mitochondrial genes should not be discarded a priori from phylogenetic analysis and that sophisticated analytical treatment, particularly with respect to third codon positions in protein-encoding genes, can reveal underlying phylogenetic signal (Plazzi et al., 2011).

We concur with Plazzi et al. (2011) that mitochondrial genes can be highly informative markers, particularly with respect to shallow nodes. Empirical phylogenetic studies of bivalves already actively sample mitochondrial genes (both ribosomal and protein-encoding) in addition to their nuclear counterparts, and for resolving various taxonomic ranks (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Campbell et al., 2005; Giribet et al., 2006; Kappner and Bieler, 2006; Mikkelsen et al., 2006; Wood et al., 2007; Tëmkin, 2010; Wilson et al., 2010). However, the second claim, that sophisticated analytical treatment of mitochondrial genes can unearth phylogenetic signal among deep divergences, is dubious

for two reasons. First, the approach taken by Plazzi et al. (2011) with respect to evaluating phylogenetic utility is to test complex models and an assortment of partitioning schemes for describing their dataset, and to obtain an optimal set of model parameters. But they do not evaluate phylogenetic utility in the context of previous phylogenetic studies, particularly those that have implemented both nuclear and mitochondrial genes together. Consequently, it is clarified neither why the mitochondrial data are so markedly discordant with respect to other multilocus data, nor how reconciliation is possible. As a corollary, it is also not clarified why exclusive use of mitochondrial data should be preferred over nuclear data, given the significant topological discord that they engender.

Second, a number of studies has previously demonstrated topological incongruence between mitochondrial and nuclear datasets (e.g., Degnan, 1993; Slade et al., 1994; reviewed by Ballard and Whitlock, 2004). Although algorithmic approaches can elucidate phylogenetic signal, over-parameterization of nucleotide and codon models can reduce their predictive power, or even engender problems of non-identifiability, in addition to artificially inflating nodal support (Chang, 1996; Steel, 2005). This could partially explain why the approach of analyzing the same four mitochondrial genes yielded radically different topologies upon the addition of taxa, with almost flawless nodal support values in the more recent study (Plazzi and Passamonti, 2010, *contra* Plazzi et al., 2011).

Phylogenomic assessments of molluscan relationships shed little further light on basal relationships, beyond favoring the mutual monophyly of Protobranchia and Autobranchia. This is a consequence of limitations in sampling of Autobranchia, whose internal relationships were not the focus of these studies. For example, Kocot et al. (2011) did not include representatives of Nuculanida, Archiheterodonta, or Anomalodesmata. Similarly,



**Figure 1.1.** Phylogenetic hypotheses of higher bivalve relationships proposed by different authors. (A) Purchon (1987) based on phenetic analysis of morphological data. (B) Waller (1990, 1998) based on non-numerical cladistic analyses of morphology. (C) Proposed evolutionary tree of Morton (1996). (D) Salvini-Plawen and Steiner (1996), parsimony analysis of morphological data. (E) Suggested evolutionary tree of Cope (1997). (F) Giribet and Wheeler (2002), based on the parsimony analysis of morphology and three molecular markers (18S rRNA, 28S rRNA, COI). (G) Giribet and Distel (2003), based on parsimony analysis of four molecular markers (18S rRNA, 28S rRNA, COI, histone H3). (H) Synoptic classification of Bieler et al. (2010). (I) Plazzi and Passamonti (2010), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). (J) Wilson et al. (2010), based on Bayesian analysis of five molecular markers (16S rRNA, 18S rRNA, 28S rRNA, COI, histone H3). (K) Synoptic classification of Carter et al. (2011). (L) Plazzi et al. (2011), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). Colors in tree topology correspond to major lineages (red: Protobranchia; green: Pteriomorpha; orange: Palaeoheterodonta; indigo: Archiheterodonta; purple: Anomalodesmata; blue: remaining Euheterodonta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Smith et al. (2011) did not sample Archiheterodonta or Anomalodesmata, and both studies used a previously published small EST data set for a single exemplar of Palaeoheterodonta. This may in part explain the counterintuitive sister relationship of Pteriomorphia and Euheterodonta (to the exclusion of Palaeoheterodonta) obtained by Smith et al. (2011), although this result was not supported under some algorithmic treatments

To redress the topological conflict caused by the use of exclusively mitochondrial datasets versus datasets that include nuclear genes, as well as to test hypotheses of basal relationships, we investigated the phylogeny of bivalves using four nuclear, protein-encoding loci: ATP synthase *b*, elongation factor-1 $\alpha$ , myosin heavy chain type II, and RNA polymerase II. The study included representatives of all major bivalve lineages. We performed a phylogenetic analysis of nucleotide sequence data, manipulating the treatment of length variability and/or third codon positions to investigate phylogenetic robustness. From the topologies obtained, we reexamined hypotheses of basal relationships and evaluated these four molecular markers as predictors of bivalve phylogeny.

## **Materials and methods**

### *Taxon sampling*

Specimens for the study were obtained as part of the Bivalve Tree of Life (BivAToL) project (where most will also serve as exemplar species in separate analyses of other molecular loci, as well as morphological characters); additional sequence data were obtained from the Protostome Tree of Life project (see project information in Acknowledgments), or accessed

from GenBank. The 45 ingroup taxa sampled consisted of 5 Protobranchia, 14 Pteriomorpha, 3 Palaeoheterodonta, 2 Archiheterodonta, 3 Anomalodesmata, and 18 other Euheterodonta. Outgroup taxa for the study consisted of 7 gastropods, 1 chiton, 1 scaphopod, and 2 cephalopods. However, we observed that the highly divergent sequences of all outgroups except Gastropoda resulted in non-monophyly of the ingroup (Supplementary Fig. 1.S1). Given that bivalve monophyly has been demonstrated recently using phylogenomic approaches (Smith et al., 2011) and this study is concerned only with internal relationships, we limited the outgroup sampling to a subset of gastropods for our principal analyses. The full list of taxa included in our study is provided in Supplementary Table 1.S1.

#### *Molecular methods*

Total RNA was isolated from tissues preserved in RNAlater® (Ambion) or frozen at -80 °C, using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed with 1–2 mg of total RNA using the RETROscript™ kit (Ambion). Sequence data were obtained using degenerate PCR primers, whose sequences and original references are provided in Table 1.1.

Fragments of the head portion of myosin heavy chain type II and elongation factor-1a were amplified from template cDNA following nested PCR reaction protocols, described by Aktipis and Giribet (2010). Fragments of RNA polymerase II were amplified following protocols described by Regier and Shultz (2000). ATP synthase b fragments were amplified from cDNA following touchdown PCR reaction protocols modified from Sperling et al. (2007). Initial touchdown reactions amplified fragments using the external primers ATPbF and ATPbR, in a 25 IL reaction (1 IL cDNA template, 20.5 IL ddH<sub>2</sub>O, 2.5 IL AmpliTaq™

10x PCR buffer, 0.5 IL dNTPs [10  $\mu\text{mol L}^{-1}$ ], 0.25 IL of each primer [100  $\mu\text{mol L}^{-1}$ ], and 0.625 U AmpliTaq™ enzyme) with the following parameters: 1 min of denaturation at 94 °C, 45 s of annealing at 52 °C for two cycles, then annealing temperature was lowered one degree every two cycles, terminating at 40 °C for a total of 26 cycles, followed by

**Table 1.1.** List of primer sequences used for PCR amplification and sequencing with original references.

Myosin heavy chain		
mio3	5' -GGN GTN YTN GAY ATH GC-3'	Ruiz-Trillo et al. (2002)
mio4	5' -GGR AAN CCY TTN CKR CAD AT-3'	Ruiz-Trillo et al. (2002)
mio6	5' -CCY TCM ARY ACA CCR TTR CA-3'	Ruiz-Trillo et al. (2002)
mio7	5' -TGY ATC AAY TWY ACY AAY GAG-3'	Ruiz-Trillo et al. (2002)
ATP synthase subunit B		
ATPbF	5' -GTN GAY GTN CAR TTY GAY GA-3'	Sperling et al. (2007)
ATPbR	5' -CYA TYT TGG GTA TGG ATG AA-3'	Sperling et al. (2007)
ATPb200F	5' -NCC NAC CAT RTA RAA NGC-3'	This study
ATPb1088R	5' -RTW GGD GAM CCA ATT GAY GA-3'	This study
Elongation factor-1a		
RS2F (52.4F)	50 TCN TTY AAR TAY GCN TGG GT-30	Regier and Shultz (1997)
RS4R (52RC)	5' -CCD ATY TTR TAN ACR TCY TG-3'	Regier and Shultz (1997)
RS3F (45.71F)	5' GTN GSN GTI AAY AAR ATG GA-3'	Regier and Shultz (1997)
RS6R (53.5RC)	5' ATR TGV GMI GTR TGR CAR TC-3'	Regier and Shultz (1997)
RNA polymerase II		
15F	5' -ACW GCH GAR ACH GGK TAY ATY CA-	Shultz and Regier (2000)
14F	5' -YTK ATH AAR GCT ATG GA-3'	Shultz and Regier (2000)
17R	5' -TTY TGN GCR TTC CAD ATC AT-3'	Shultz and Regier (2000)

45 s of annealing at 52 °C for 10 cycles, and a 2 min extension at 72 °C, with a final 7 min extension at 72 °C. This initial PCR product was then amplified in a second PCR reaction using the internal primers ATPb200F and ATPb1088R, in a 50 IL reaction (2 IL touch-down template, 41 IL ddH<sub>2</sub>O, 5.0 IL AmpliTaq™ 10x PCR buffer, 1 IL dNTPs [10  $\mu\text{mol L}^{-1}$ ], 0.5 IL of each primer [100  $\mu\text{mol L}^{-1}$ ], and 1.25 U AmpliTaq™ enzyme, Applied Biosystems, Carlsbad, CA, USA) with the following parameters: initial 2 min denaturation at 94 °C, then 35 cycles of 30 s of denaturation at 94 °C, 45 s of annealing at 52 °C, and 2 min extension at



72 °C, with a final 7 min extension at 72 °C. Annealing temperatures for ATP b ranged between 46 °C and 55 °C.

All amplified samples were purified using an Eppendorf vacuum (Hamburg, Germany) and Millipore Multiscreen® PCR196 cleanup filter plates (Billerica, MA, USA) following the manufacturers' instructions. Sequencing was performed in a GeneAmp® PCR system 9700 (Perkin Elmer, Waltham, MA, USA) using ABI PRISM™ BigDye™ v.3 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and following the manufacturer's protocol. The BigDye-labeled PCR products were cleaned with Performa® DTR v3 96-well short plates (Edge BioSystems, Gaithersburg, MD, USA) and directly sequenced using an automated ABI Prism® 3730 Genetic Analyzer.

Chromatograms obtained from the automatic sequencer were analyzed using the sequence editing software Sequencher™4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). External and internal primer regions were removed from these edited sequences. The lengths of the amplicons for each gene are provided in Supplementary Table 1.S2.

### *Phylogenetic analysis*

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted on static alignments, which were inferred as follows. Length variable sequences (elongation factor-1a and myosin) were converted to amino acid sequences, which were aligned using MUSCLE ver. 3.6 (Edgar, 2004) with default parameters. The resulting amino acid alignments were used to guide the alignment of the corresponding nucleotide sequences. Length invariable sequences (ATP synthase b and RNA polymerase II) were confirmed using amino acid sequence translations to ensure that no treatment with MUSCLE ver. 3.6 was

required. These four data partitions constituted the full dataset (henceforth Dataset 1). Length variable data partitions (elongation factor-1a and myosin) were subsequently treated with GBlocks v. 0.91b (Castresana, 2000) to cull positions of ambiguous homology from amino acid sequence alignments. We allowed less strict flanking positions for both genes, as use of this feature retains the conserved positions at the beginnings and ends of each dataset (disuse of this feature affected only the termini of the amino acid alignments). Together with the length invariable data partitions, these formed a second dataset free of length variability entirely (henceforth Dataset 2).

We removed the third codon positions of the full dataset to form a third dataset, wherein only first and second codon positions were retained in addition to some length variability (henceforth Dataset 3). Finally, we also removed the third codon positions of Dataset 2 to form the smallest dataset, wherein only 1st and 2nd codon positions, but no length variable regions, were retained (henceforth Dataset 4). The lengths of the aligned datasets in each treatment are indicated in Supplementary Table 1.S2. Aligned datasets are available upon request from the authors.

BI analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2005) on 12 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu), with a unique model of sequence evolution with corrections for a discrete gamma distribution and/or a proportion of invariant sites specified for each partition, as selected in jModeltest ver. 0.1.1 (Posada, 2008; Guindon and Gascuel, 2003) under the Akaike Information Criterion (Posada and Buckley, 2004). Model recommendations for each dataset are indicated in Supplementary Table 1.S2. Default priors were used starting with random trees, and three runs, each with three hot and one cold Markov chains, were

performed for all four datasets until the average deviation of split frequencies reached  $<0.01$  (107 generations). Stationarity was checked using Tracer ver. 1.5 (Rambaut and Drummond, 2009). After burn-in samples were discarded, sampled trees were combined in a single majority-rule consensus topology, and the percentage of trees in which a node was recovered was taken as the posterior probability for that node.

ML analyses were conducted using RAxML ver. 7.2.7 (Stamatakis, 2006) on 24 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu). For the maximum likelihood searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + C) was specified for each data partition, and 250 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (250 replicates) using the GTR-CAT model (Stamatakis et al., 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches.

A Shimodaira–Hasegawa (SH) test was conducted using RAxML ver. 7.2.7. We enforced an ingroup topology consistent with the Amarsipobanchia hypothesis (sensu Plazzi et al., 2011) and compared it to the ML topology obtained using Dataset 1. To generate the null distribution, 500 resampling replicates were conducted.

## **Results**

Runs of MrBayes ver. 3.1.2 generally reached stationarity in ca. 106 generations; 2 x 106 generations (20%) were hence discarded as burn-in. One of the three runs for Dataset 4 became trapped on a local optimum and was therefore abandoned after the remaining two

runs converged. BI analysis of all datasets recovered the monophyly of all six major lineages except Palaeoheterodonta, and basal relationships as follows: (Protobranchia (Pteriomorphia ((Archiheterodonta + Palaeoheterodonta) (Euheterodonta)))) (Fig. 1.2). Datasets 1–3 also recovered a basal dichotomy between Anomalodesmata and the remaining Euheterodonta, but Dataset 4 recovered Anomalodesmata nested within Euheterodonta (with a lineage of Cardioidea as sister to the remaining Euheterodonta). Derived relationships within Pteriomorphia and Euheterodonta were generally unstable, with numerous superfamilies recovered as non monophyletic (Fig. 1.3).

ML analysis using RAxML ver. 7.2.7 resulted in tree topologies with  $\ln L = -44419.380$ ,  $\ln L = -42102.014$ ,  $\ln L = -15746.721$ , and  $\ln L = -14510.373$  for Datasets 1–4, respectively. ML topologies of each dataset were almost identical to those obtained by corresponding BI analyses. However, Dataset 1 recovered the monophyly of all six major lineages, whereas Datasets 2–4 favored the paraphyly of Palaeoheterodonta with respect to Archiheterodonta. All analyses recovered a sister group relationship of Palaeoheterodonta and Archiheterodonta, and therefore non-monophyly of Heterodonta. As in the BI topologies, the sister relationship of Anomalodesmata to the remaining Euheterodonta was obtained by all datasets except Dataset 4 (Fig. 1.2).

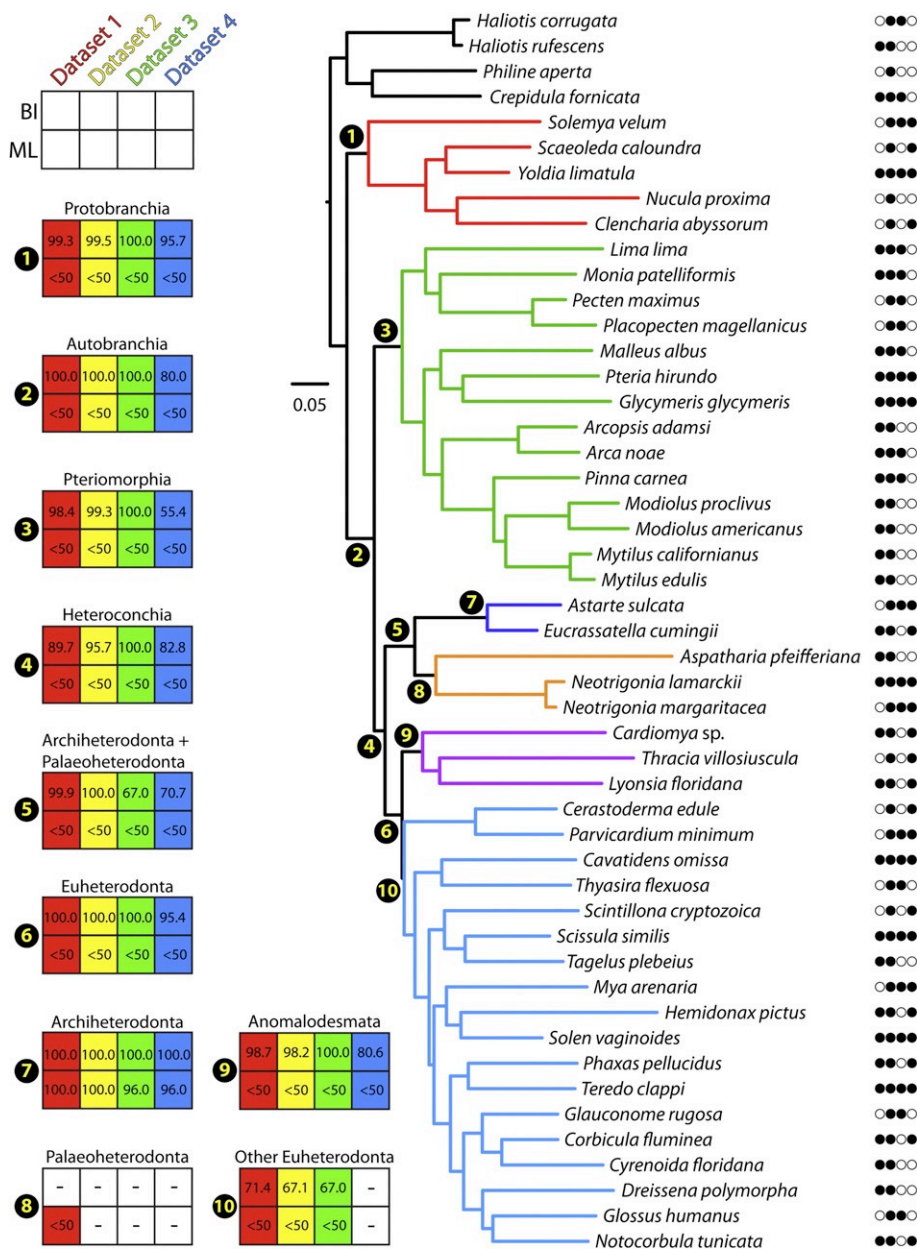
Removal of the variable third codon positions is expected to limit phylogenetic inference among shallow nodes. Accordingly, Datasets 3 and 4 resulted in non-monophyly of one or more congeneric species pairs (*Modiolus* and *Mytilus*) in both BI and ML analyses. There is also a trend toward declining posterior probabilities among BI topologies upon removal of both third codon positions and length variability. Bootstrap resampling frequencies across all four ML topologies were limited. A strict consensus of all tree

topologies obtained, underscoring those nodes consistently found across the analytical space, is shown in Fig. 1.4. Salient points are the monophyly of Protobranchia, Autobranchia, Pteriomorphia, Heteroconchia, Palaeoheterodonta + Archiheterodonta, Euheterodonta and Anomalodesmata.

The SH test comparing the ML topology (obtained with Dataset 1;  $\ln L = -44419.380$ ) to the topology consistent with the Amarsipobranchia hypothesis ( $\ln L = -44653.593$ ) recovered a difference in log likelihood of 234.20 (standard deviation of 30.19), and rejected the null hypothesis of equal likelihood of the two topologies.

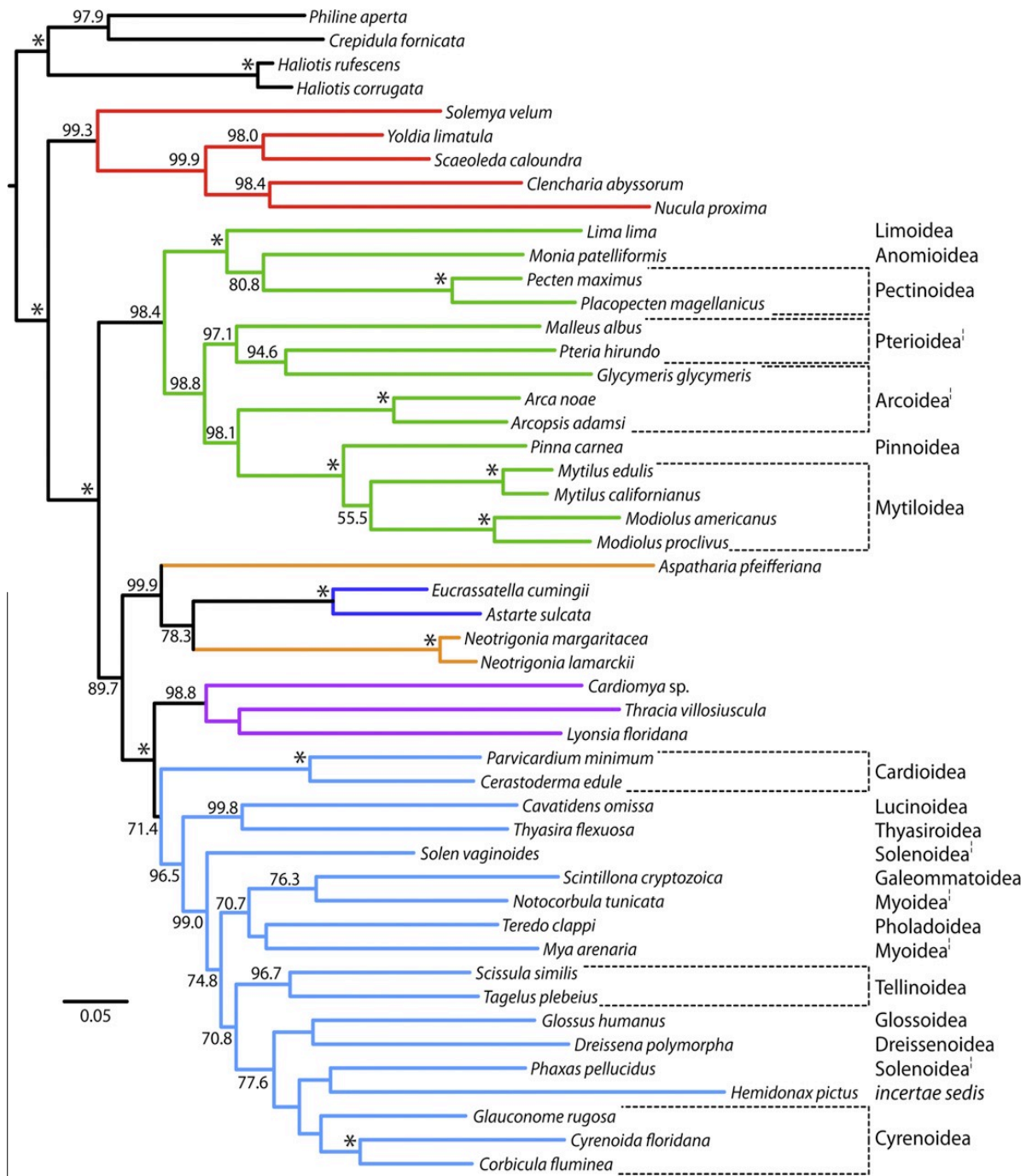
## Discussion

Exclusive use of phylogenetic data from one source—nuclear, mitochondrial, plastid, or any other—can engender biases in tree topology. These biases can only be tested by comparing topological congruence from other data sources. Topological discordance between mitochondrial and nuclear datasets was observed early in the history of molecular phylogenetics (e.g., Degnan, 1993; Slade et al., 1994) and is attributable to multiple idiosyncrasies of the mitochondrial genome. For example, the length of the mitochondrial genome constitutes only a minuscule fraction of the length of the nuclear haploid genome—approximately 0.0073% in the case of the gastropod *Lottia gigantea* G.B. Sowerby, I, 1834 (rv26,400 bp in the mitochondrial genome, compared to ca. 359.5 Mbp in the nuclear genome). Mitochondrial and nuclear genomes also differ in degree of recombination, modes of inheritance, and incidence of introns—differences that can affect inferences of evolutionary history. Additionally, mutation rates of mitochondrial DNA are generally higher



**Figure 1.2.** Phylogenetic relationships of Bivalvia based on maximum likelihood analysis of four nuclear protein-encoding genes (ln L = -44419.380). Colors in tree topology correspond to major lineages (as in Fig. 1). Navajo rugs correspond to 10 nodes of interest. Colors in Navajo rugs correspond to each dataset; numbers in Navajo rugs indicate posterior probabilities from Bayesian analysis (top row) or bootstrap resampling frequency (bottom row). Failure to retrieve a node is indicated as a white entry (without a number indicating nodal support). Filled circles at the right of each taxon indicate representation by the gene of interest, from left to right: ATP synthase b, elongation factor-1a, myosin heavy chain type II, and RNA polymerase II. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than those of nuclear DNA, with typical estimates ranging from two to over tenfold with respect to nuclear genes (Brown et al., 1979; Moriyama and Powell, 1997; Denver et al., 2000; Lambert et al., 2002; Howell et al., 2003), limiting inference of deep phylogenetic events, namely, the origin and early diversification of bivalves. Strand-specific substitution biases have also been shown to occur in mitochondrial genomes (Ballard, 2000; Haag-Liautard et al., 2008). Consequently, a number of reviews have critiqued the role of mitochondrial DNA in phylogenetics, suggesting caution in their use, simultaneous deployment with nuclear genes, and/or omission from phylogenetic study altogether (e.g., Ballard and Whitlock, 2004; Rubinoff and Holland, 2005; Fisher-Reid and Wiens, 2011). These concerns weigh heavily upon recent inferences of bivalve basal relationships based solely on mitochondrial genes (Plazzi and Passamonti, 2010; Plazzi et al., 2011). As proponents of the total evidence approach, we do not countenance in principle or in practice the omission of mitochondrial genes from assessment of phylogenetic relationships. Previous studies have demonstrated the utility of mitochondrial genes for resolving shallow nodes in bivalve phylogenies, a property stemming from the variability and increased mutation rate of the mitochondrial genome (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Wilson et al., 2010)—the very property that discourages their use for resolving deep nodes. Moreover, as algorithms and models are improved, especially for analyzing mitochondrial gene order and amino acid sequence data, the utility of mitochondrial genes is anticipated to increase with improved taxonomic sampling. For this reason, we continue to advocate the inclusion of mitochondrial genes in concert with nuclear ones in order to resolve relationships of various phylogenetic depths. Accordingly, we regard with skepticism a topology of basal bivalve relationships that is derived exclusively from a particular genomic source with a high



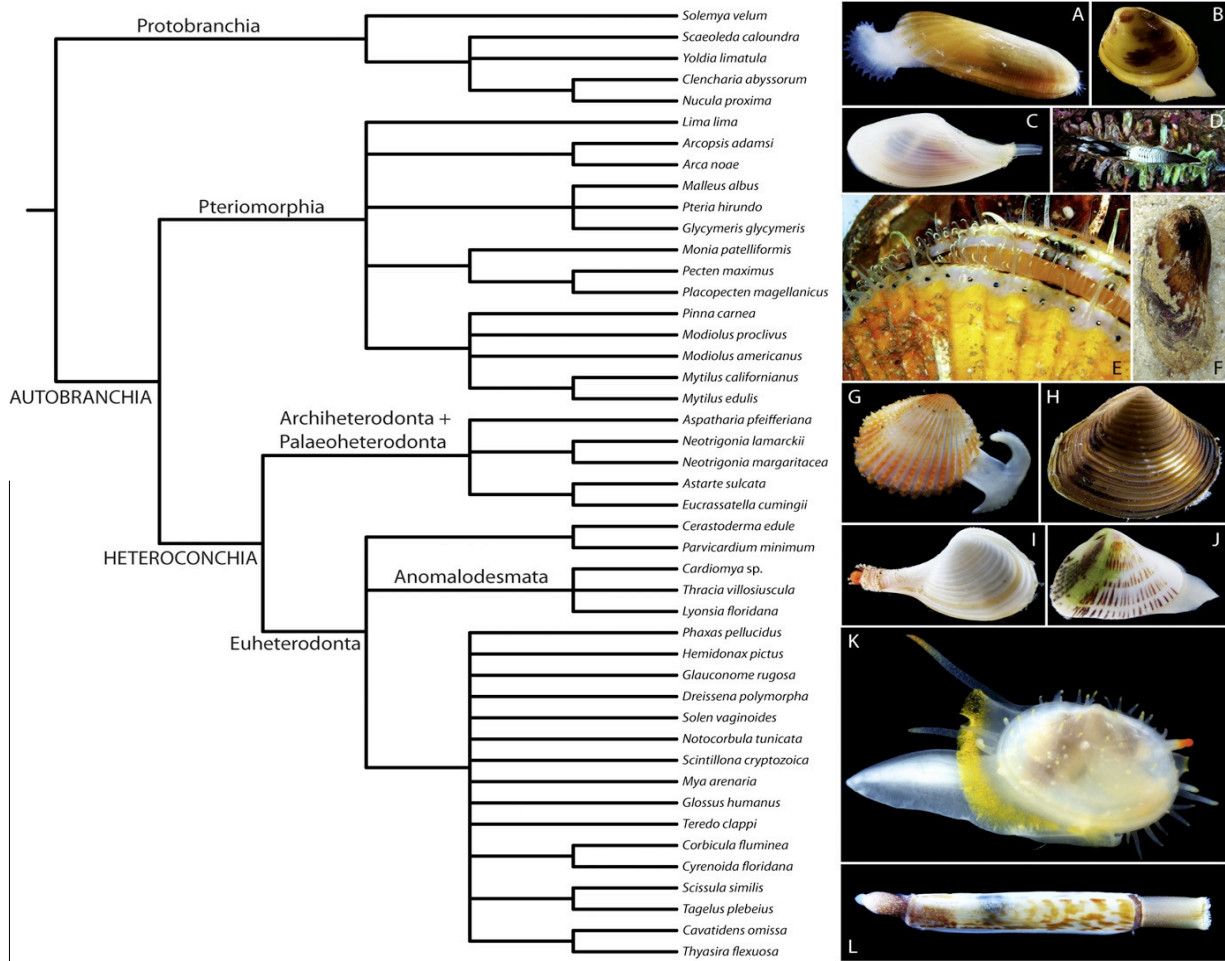
**Figure 1.3.** Phylogenetic relationships of Bivalvia based on Bayesian inference analysis of four nuclear protein-encoding genes. Colors in tree topology correspond to major lineages (as in Fig. 1.1). Numbers on nodes indicate posterior probabilities, with asterisks indicating a value of 100%. Superfamilies of Pteriomorpha and Euheterodonta (not including Anomalodesmata) are as indicated, with a broken line symbol indicating non-monophyletic groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



mutation rate and concomitant complex algorithmic treatment that yields uniformly high nodal support. The topology supporting Amarsipobranchia (sensu Plazzi et al., 2011) is based on four mitochondrial genes and suggests radical reorganization of bivalve higher-level systematics. In addition, the use of multiple protein-encoding sequences with single nucleotide indels among the datasets of Plazzi et al. (2011) is particularly suspect; the authors admitted to being unable to rule out sequencing errors or numts as potential explanations, but nevertheless favored the functionality of their sequenced amplicons.

Separately, we observe that the contending hypotheses of basal bivalve relationships (Giribet and Wheeler, 2002; Wilson et al., 2010) are based on multilocus datasets, but those dominated by nuclear ribosomal genes. These datasets have previously employed the “workhorses” of molecular phylogenetics: the mitochondrial genes 16S rRNA and cytochrome c oxidase subunit I; the nuclear ribosomal genes 18S rRNA and 28S rRNA; and a small (327-bp) fragment of the nuclear protein-encoding gene histone H3. This combination of nuclear and mitochondrial genes was anticipated to be capable of resolving relationships of various depths, by sampling both slow (e.g., 18S rRNA) and fast evolving sites (e.g., 16S rRNA). However, the amplicons of these genes are not uniformly distributed in length; the two nuclear ribosomal genes alone typically constitute ca. 70% of the entire dataset (e.g., Wilson et al., 2010). Therefore, evidence of misleading signal stemming from disproportionate representation of nuclear ribosomal genes must also be tested independently of mitochondrial gene signal.

Here we have reevaluated basal bivalve relationships using a separate set of genes altogether to redress the topological conflicts in early bivalve phylogenies. Our four molecular loci are not inherited as a linked or structurally dependent unit, as are the



**Figure 1.4.** Strict consensus of all eight topologies obtained (two from each dataset). Bivalve exemplars photographed are: (A) *Solemya velum* (Protobranchia); (B) *Nucula expansa* Reeve, 1855 (Protobranchia); (C) *Scaolea caloundra* (Protobranchia); (D) *Pinna rudis* Linnaeus, 1758 (Pteriomorpha); (E) *Aequipecten opercularis* (Linnaeus, 1758) (Pteriomorpha), detail of simple eyes; (F) *Modiolus proclivus* (Pteriomorpha); (G) *Neotrigonia lamarckii* (Palaeoheterodonta); (H) *Eucrassatella cumingi* (Archiheterodonta); (I) *Cuspidaria latesulcata* (Tenison-Woods, 1878) (Anomalodesmata); (J) *Hemidonax pictus* (Euheterodonta); (K) *Scintillona cryptozoica* (Euheterodonta); (L) *Solen vaginoides* (Euheterodonta).

mitochondrial genes. The tree topologies that we obtained are remarkably congruent with the traditional classification of bivalves (sensu Bieler and Mikkelsen, 2006). Although bootstrap resampling frequencies were low in ML analyses, all topologies examined recovered the monophyly of Protobranchia, Autobranchia, Heteroconchia, Pteriomorpha, Archiheterodonta, and Euheterodonta (including a monophyletic Anomalodesmata) (Figs.

1.2–4). Within Euheterodonta, we obtained a sister relationship of Anomalodesmata to the remaining Euheterodonta in the majority of topologies examined. A basal position of Anomalodesmata among Euheterodonta accords with previous hypotheses based on morphological and molecular characters (e.g., Giribet and Wheeler, 2002; Harper et al., 2006; Taylor et al., 2007).

Among derived Euheterodonta, we observe with interest the clade consisting of *Corbicula fluminea*, *Cyrenoida floridana*, and *Glaucanome rugosa* (Figs. 1.2 and 1.3), insofar as exemplars of the same three genera were found to cluster in a separate phylogenetic analysis using nuclear ribosomal markers (Taylor et al., 2009). This clade, comprising the superfamilies Cyrenoidea and Cyrenoidoidea, was used to justify the further dismantling of Lucinoidea by Taylor et al. (2009), wherein Cyrenoididae had been placed previously. Our analyses thus corroborate the exclusion of cyrenoidids from Lucinoidea, with support (PP = 99.0%, 98.1%, 95.4%, and 97.0% in Datasets 1–4, respectively), but places Cyrenoididae as sister group to Cyrenidae (formerly Corbiculidae), with Glauconomidae as their sister family, therefore making Cyrenoidea paraphyletic with respect to Cyrenoidoidea.

Palaeoheterodonta was obtained as monophyletic in only one of eight topologies examined. The recovery of a paraphyletic Palaeoheterodonta with respect to Archiheterodonta appears to stem from missing data for the taxon *Aspatharia pfeifferiana*, which is represented here by only two gene partitions (Fig. 1.2, Supplementary Table 1.S1). We observe similar sensitivity to analytical treatment among other taxa that are represented by fewer sequence data, visualized as little structure within Pteriomorphia and Euheterodonta subsequent to strict consensus across all topologies examined (Fig. 1.4). The monophyly of Palaeoheterodonta is contentious (Purchon, 1987; Morton, 1996; Salvini-Plawen and Steiner

1996; Waller, 1998; Cope, 2000) and we are as yet unable to test it, given our present sampling of only two of the 175 extant palaeoheterodont genera (Roe and Hoeh, 2003). Similarly, we observe extensive non-monophyly among the constituent superfamilies of Pteriomorphia and Euheterodonta that were sampled in this study (Fig. 1.3), but the internal relationships of these diverse subclasses is beyond the scope of the present study.

We also uniformly obtain the monophyly of the curious clade (Archiheterodonta + Palaeoheterodonta), a result obtained by a previous phylogenetic analysis (using the aforementioned “workhorses”; Wilson et al., 2010) (Figs. 1.2–4). In addition to phylogenetic support and stability based on molecular data, this sister relationship is potentially supported by morphological synapomorphies, namely the hind end of the ctenidia unattached to the mantle (Purchon, 1990), or the presence of Atkin’s type D ciliary currents, although both characters require further scrutiny. Members of Carditida (i.e., *Cyclocardia ventricosa* and *Astarte sulcata*—see Yonge, 1969; but see Saleuddin, 1965, for a different view on *Astarte*) and Unionida show Atkin’s type D ciliary currents (see Atkins, 1937). Tevesz (1975) also considered the ctenidial ciliation of Neotrigonia to be of type D, as in Unionida, but this was disputed by Morton (1987). Further phylogenetic study is anticipated to test the monophyly of this clade using separate molecular loci, in addition to morphological characters.

In general, the removal of length variable regions and/or third codon positions from the alignment had no effect on the relationships obtained (nodes 1–7, 9 in Fig. 1.2). Bootstrap resampling frequencies were very low across all topologies, possibly as a consequence of the degree of character conflict, missing data, the short length of the combined dataset, or some combination of these. In any case, we cannot assess the effects of analytical treatment based on the bootstrap values. However, posterior probabilities in Bayesian analyses marginally

increased upon removal of length variable regions and tended to decrease for some nodes upon removal of both length variability and third codon positions. These data suggest that complexity of algorithmic treatment is not required to elucidate phylogenetic signal among these nuclear protein-encoding genes, particularly with respect to treatment of third codon positions, as suggested for mitochondrial genes (Plazzi et al., 2011).

Comparison of the consensus topology obtained in the present study to previous hypotheses of bivalve relationships indicates significant congruence with a topology using five genes (Wilson et al., 2010) with respect to relationships among Autobranchia, and with the topology recovered by second-generation sequencing techniques (Smith et al., 2011) with respect to the monophyly of Protobranchia. These results favor the traditional classification of Bivalvia (Bieler and Mikkelsen, 2006), albeit with the emended relationship of (Archiheterodonta + Palaeoheterodonta). None of these results is consistent with a group uniting Nuculanida, Pteriomorpha, Archiheterodonta, Anomalodesmata, and the other Euheterodonta, i.e., Amarsipobranchia (sensu Plazzi et al., 2011). We therefore reject the putative homology of the gill character that supposedly unites this clade, given (a) the homoplastic mapping engendered by this character upon superimposition on either our consensus topology or the topology obtained by Wilson et al. (2010)—congruent with ours but derived from a completely nonoverlapping set of genes; (b) the enforced homoplasy of other morphological characters traditionally considered synapomorphies for various groups of bivalves, if the topology of Plazzi et al. (2011) were accepted; and (c) the results of the SH test, which indicates that the Amarsipobranchia hypothesis is significantly worse than the maximum likelihood topology obtained using either the “workhorses” or the four genes analyzed in this study.

The robustness of the phylogenetic signal embedded in these four protein-encoding genes, in addition to the marked congruence observed between these markers and the “workhorses” of molecular phylogenetics (see Aktipis and Giribet, 2010, 2012, for a similar case in gastropods), strongly favors their continued use in the study of molluscan phylogenetics specifically, and invertebrate systematics generally. Regrettably, we were unable to sample more broadly the diversity of both Euheterodonta and Pteriomorphia, which would have enabled an assessment of the utility of these markers for elucidating relationships within diverse subclasses (Fig. 1.3), but consistent amplification across taxa can prove difficult, even when using freshly collected tissue. The utility of these markers for elucidating shallow relationships is beyond the scope of this study. However, comparable use of one or more of these loci in studies of other invertebrate taxa (e.g., Regier and Shultz, 1997, 2000; Sperling et al., 2007; Aktipis and Giribet, 2010) offers promising prospects for the applicability of these markers in multilocus datasets, albeit with the added challenge of traditional RT-PCR techniques. We additionally observe that widespread proliferation of phylogenomic data for non-model invertebrate taxa (e.g., Dunn et al., 2008; Hejnol et al., 2009; Meusemann et al., 2010; Kocot et al., 2011; Smith et al., 2011) heralds access to an unprecedented stockpile of efficacious molecular loci for phylogenetic study of diverse invertebrate groups.

## **Conclusion**

Concordance between topologies based on the “workhorses”—the five traditionally used molecular loci that include nuclear and ribosomal genes (e.g., Wilson et al., 2010)—and the

four nuclear protein-encoding markers employed here suggest that robust assessment of phylogenetic relationships of various depths is best achieved by sampling markers spanning a spectrum of evolutionary rates.

### **Author contributions**

PPS: Designed, conducted phylogenetic analyses; wrote manuscript; made figures.

VLG: Collected fresh tissues for RNA extraction; collected nucleotide sequence data; designed bivalve-specific primers for ATP Synthase beta (Table 1.1); generated and checked alignments; accessioned sequences in GenBank; wrote Molecular Methods section; edited manuscript.

GYK: Generated and checked alignments; conducted likelihood ratio tests using jModeltest; edited manuscript.

SCSA: Collected nucleotide sequence data; approved manuscript. AG: Collected fresh tissues for RNA extraction; collected nucleotide sequence data.

TMC, EAG, EMH, JMH, PMM, JDT: Collected fresh tissues for RNA extraction; read and approved manuscript.

RB: Conceived and supervised Bivalve Tree of Life project; collected fresh tissues for RNA extraction; wrote and checked discussion on most recent systematics of bivalves; edited manuscript.

GG: Conceived and supervised Bivalve Tree of Life project; collected fresh tissues for RNA extraction; supervised molecular work; edited manuscript.

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## **Appendix 1. A. Supplementary material**

Supplementary data associated with this article can be found, in the online version, at

<http://dx.doi.org/10.1016/j.ympev.2012.05.025>.

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## CHAPTER 2

### **Transcriptomic-scale sequencing engenders congruence in morphology and molecules: Phylogenomic resolution of relationships within Bivalvia (Mollusca)**

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## Abstract

Bivalves represent the second largest living class of molluscs, following gastropods, and their internal phylogenetic relationships have been debated for decades. Although recent investigations have revealed general agreement on the phylogenetic placement of the major bivalve lineages, support for several critical basal nodes is lacking, and strong incongruence has been shown between mitochondrial and nuclear gene-based phylogenies. Phylogenomic approaches have recently been employed to obtain robust resolution of molluscan relationships, but conflict in morphological and molecular datasets persists within some major molluscan lineages, particularly Bivalvia. Here we provide the first phylogenomic analysis of Bivalvia aimed at resolving deep divergences within this group to have a better framework for understanding this diverse and fossil-rich group of animals. All six major lineages of bivalves (Archiheterodonta, Anomalodesmata, Inaequidonta, Palaeoheterodonta, Protobranchia, and Pteriomorphia) were sampled resulting in 31 newly sequenced Illumina-based bivalve transcriptomes, in addition to six transcriptomes from representatives of other molluscan lineages. This constitutes the most comprehensive phylogenomic dataset to date for inferring deep relationships within Bivalvia. Subsequent analyses obtained robust resolution of bivalve major lineages, largely corroborating classical taxonomic relationships based mostly on paleontological and neontological morphological data. This includes the monophyly of Autobranchia, Pteriomorphia, Heteroconchia, Palaeoheterodonta, Heterodonta, Archiheterodonta, Euheterodonta and Inaequidonta. Although protobranchians remain sensitive to analytical treatment, our main clade of interest, Archiheterodonta is well supported as the sister group of Euheterodonta.

## Introduction

Recent phylogenomic approaches have been employed to explore deep molluscan relationships (Kocot *et al.* 2011; Smith *et al.* 2011), but conflicts between morphological and molecular datasets, and especially, between types of molecular data, persist within major molluscan lineages, particularly Bivalvia. The former studies employing next-generation sequencing (NGS) techniques, which include phylogenomic reconstructions with large molecular sequence datasets, recover the monophyly of bivalves and place bivalves as sister group to gastropods (Kocot *et al.* 2011) or to a gastropod + scaphopod clade (Smith *et al.* 2011), but do not really address internal bivalve relationships.

Taxonomic classifications of Bivalvia have been subjected to many revisions and reorganizations owing to the discordant phylogenetic signal from previously analyzed morphological, paleontological and molecular datasets. Notwithstanding that some molecular analyses have failed to recover the monophyly of Bivalvia, recent reconstructions recover the once ambiguous clade with robust support (Giribet & Wheeler 2002; Wilson *et al.* 2010; Kocot *et al.* 2011; Plazzi *et al.* 2011; Smith *et al.* 2011). Though taxonomic ranking and stability have not been established for all bivalve lineages, six major clades are now recognized. Protobranchia comprises a group of exclusively marine bivalves having small shells characterized by varying degrees of hinge dentition and simple ctenidial structure (Giribet & Distel 2003). Pteriomorpha are found in both freshwater and marine habitats, and include the commercially important oysters and mussels (Giribet & Wheeler 2002). Morphologically they are characterized by relatively large, compressed shells, which can be smooth or ribbed and are predominantly anchored to substrates using byssal threads or valve cementation (Bieler & Mikkelsen 2006).

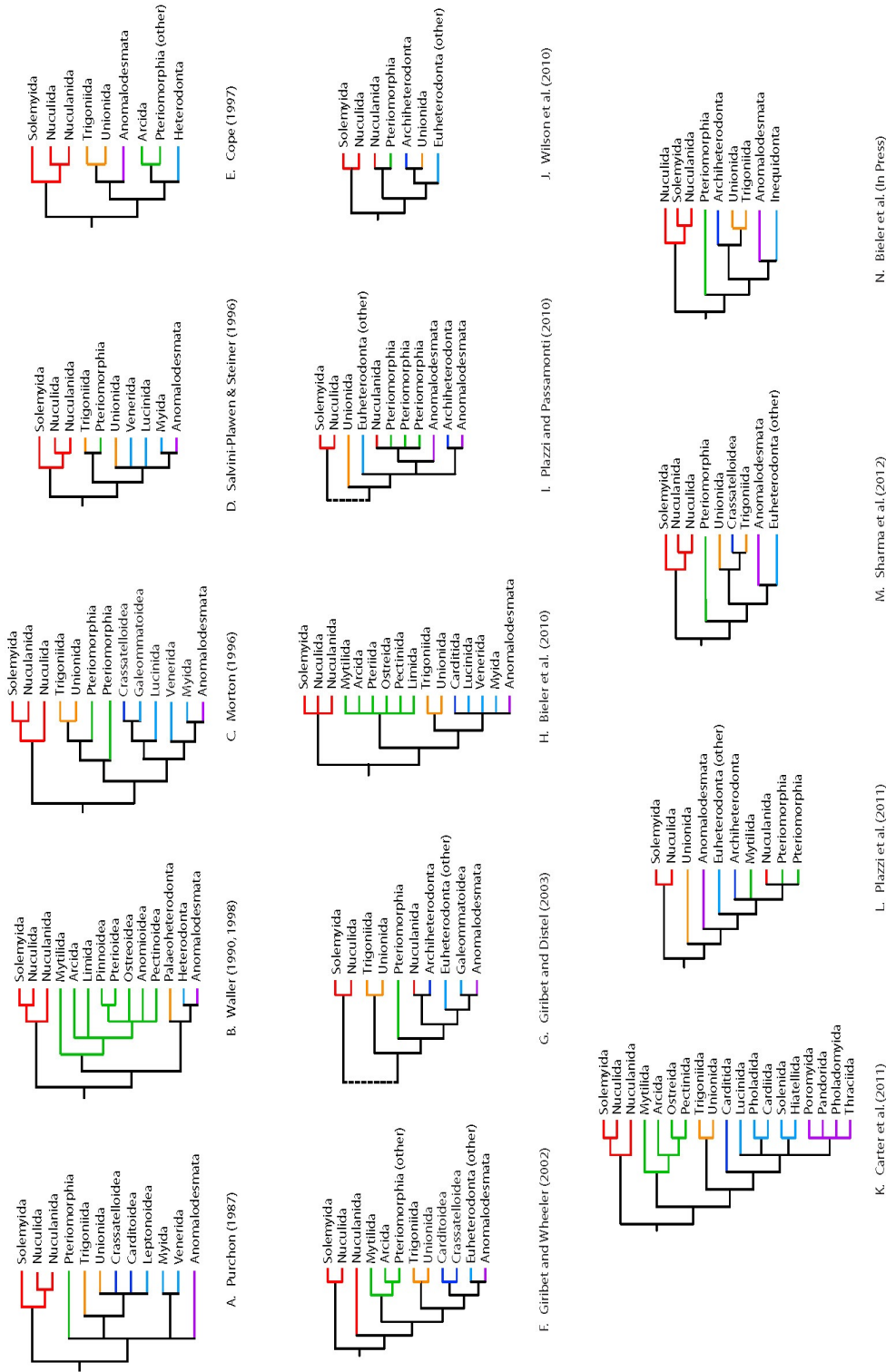
Palaeoheterodonta are both marine and limnic, with larger, often grooved shells that can be ribbed or smooth and includes freshwater mussels (Giribet & Wheeler 2002). Heterodonta includes groups such as clams, cockles, and comprises two clades: Archiheterodonta, whose members share the presence of a unique hemoglobin, and Euheterodonta, divided into Anomalodesmata and Inaequidonta. Inaequidonts, the most diverse subclade of Euheterodonta, are found both in marine and limnic environments, have the widest range of shell sizes among all bivalve groups, can have smooth or sculptured valves, and have well developed siphons (Giribet & Distel 2003; Taylor *et al.* 2007). Lastly, comprising Euheterodonta along with Inequidonta, Anomalodesmata was formerly treated as its own subclass (e.g., Newell 1965) having a highly variable shell shape and size, yet united by reduced hinge dentition and include the carnivorous bivalves (Bieler & Mikkelsen 2006; Harper *et al.* 2009).

Taxonomic relationships have been investigated for many internal bivalve clades, as in Protobranchia (Sharma *et al.* 2013), Pteriomorphia (Canapa *et al.* 2000; Matsumoto & Hayami 2000; Steiner & Hammer 2000; Matsumoto 2003; Tëmkin 2006, 2010; Waller 2006), Unionoida (Hoeh *et al.* 1999; Graf 2000; Graf & Ó Foighil 2000; Huff *et al.* 2004; Graf & Cummings 2006), Anomalodesmata (Harper *et al.* 2000; Dreyer *et al.* 2003; Harper *et al.* 2006), and the broader Heterodonta (Canapa *et al.* 1999, 2001, 2003; Park & Ó Foighil 2000; Campbell *et al.* 2004; Williams *et al.* 2004; Taylor *et al.* 2005, 2007, 2009, 2011; Mikkelsen *et al.* 2006; Taylor & Glover 2006). Recent investigations reveal general congruence in the phylogenetic placement of most major bivalve lineages, with the exception of Protobranchia and Archiheterodonta. In addition, recent molecular data based on mitochondrial genes (e.g., Plazzi *et al.* 2010, 2011, 2013) have proposed relationships that are at odds with previously published work based on ribosomal genes and morphology, and with more recent phylogenetic work based on nuclear

genes (e.g., Sharma *et al.* 2012). Insofar, two major clades of the “backbone” of bivalve evolution have yet to converge on robust phylogenetic placement: Protobranchia and Archiheterodonta (Fig. 2.1; Bieler *et al.* 2013).

Recent molecular investigations have consistently recovered the once contentious monophyly of Protobranchia (Kocot *et al.* 2012; Smith *et al.* 2011; Sharma *et al.* 2012, 2013). Relationships within Protobranchia have not yet stabilized and every iteration of relationships between Solemyida, Nuculida, and Nuculanida has been proposed based on morphological information, paleontological data, or molecular sequence information (e.g., Opponobranchia [Nuculida + Solemyida] and a clade consisting of Nuculanida + Autobranchia) (Giribet & Wheeler 2002; Giribet & Distel 2003; Giribet 2008; Wilson *et al.* 2010). As of late, reconstructions of internal protobranch phylogeny recover the clade (Nuculida + Nuculanida) as the sister group to Solemyida (Sharma *et al.* 2013). In addition to molecular sequence information, several morphological synapomorphies unite this clade, including: “primitive” eponymous protobranch gill; the palp proboscides (absent in the solemyoids, likely a consequence of obligate chemosymbiosis, as with reductions of the alimentary system); and characteristic taxodont dentition (Coan *et al.* 2000).

Likewise, recent reconstructions of relationships of Bivalvia have yet to converge on the phylogenetic placement of Archiheterodonta (Giribet & Wheeler, 2002; Wilson *et al.* 2010; Plazzi & Passamonti, 2010; Carter *et al.* 2011; Plazzi *et al.* 2011; Sharma *et al.* 2012). Phylogenetic evidence for a sister group relationship between Archiheterodonta and Euheterodonta is prevalent (e.g., Campbell 2000; Park & Ó Foighil 2000; Giribet & Wheeler 2002; Dryer *et al.* 2003; Giribet & Distel, 2003; Taylor & Glover, 2006; Harper *et al.* 2006;



**Figure 2.1.** Phylogenetic hypotheses of higher bivalve relationships proposed by different authors. (A) Purchon (1987) based on phenetic analysis of morphological data. (B) Waller (1990, 1998) based on non-numerical cladistic analyses of morphology. (C) Proposed evolutionary tree of Morton (1996). (D) Salvini-Plawen and Steiner (1996), parsimony analysis of morphological data. (E) Suggested evolutionary tree of Cope (1997). (F) Giribet and Wheeler (2002), based on the parsimony analysis of morphology and three molecular markers (18S rRNA, 28S rRNA, COI). (G) Giribet and Distel (2003), based on parsimony analysis of four molecular markers (18S rRNA, 28S rRNA, COI, histone H3). (H) Synoptic classification of Bieler *et al.* (2010). (I) Plazzi and Passamonti (2010), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). (J) Wilson *et al.* (2010), based on Bayesian analysis of five molecular markers (16S rRNA, 18S rRNA, COI, histone H3). (K) Synoptic classification of Carter *et al.* (2011). (L) Plazzi *et al.* (2011), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). (M) Bieler *et al.* (2012), based on Bayesian analysis of four molecular protein coding genes (ATP  $\beta$ , Myosin 2, EF 1  $\alpha$ , RNA polymerase). (N) Bieler *et al.* (2013) in press, total evidence tree based on Bayesian analysis of nine molecular markers (28S rRNA, 18S rRNA, 16S rRNA, H3, COI, ATP  $\beta$ , EF 1  $\alpha$ , Myosin 2, RNA polymerase) and morphology. Colors in tree topology correspond to major lineages (red: Palaeoheterodonta; green: Pteriomorpha; orange: Palaeoheterodonta; indigo: Archiheterodonta; purple: Anomalodesmata; light blue: Inaequidonta). Modified from Sharma *et al.* (2012).



Archiheterodonta has been recovered in the traditional placement as the sister group to the remaining Heterodonta (Giribet & Wheeler, 2002; Carter *et al.* 2011).

Representatives of Archiheterodonta have also been proposed to be closely related to members of Pteriomorphia (Plazzi *et al.* 2011) or related to a derived group, Anomalodesmata (Plazzi & Passamonti, 2010)—these relationships were based solely on mitochondrial gene sequence information, recovering vastly different evolutionary histories for this group. Nevertheless, recent molecular and combined molecular and morphological phylogenies have begun to converge on the placement of Archiheterodonta, forming a clade with an ancient lineage of bivalves, Palaeoheterodonta, which in turn constitutes the sister group to Euheterodonta (Wilson *et al.* 2010; Sharma *et al.* 2012; Bieler *et al.* in press).

Phylogenomic reconstructions of molluscan relationships recover the mutual monophyly of Protobranchia and Autobranchia (Pteriomorphia + Palaeoheterodonta + Archiheterodonta + Euheterodonta), yet taxon sampling is limited as the aim of these studies was not to resolve the internal relationships of the molluscan classes (Kocot *et al.* 2011; Smith *et al.* 2011). Monophyly of Autobranchia in both phylogenomic reconstructions was not fully tested as Nuculanoida, Archiheterodonta, or Anomalodesmata, were not sampled in Kocot *et al.* (2011) and Archiheterodonta or Anomalodesmata were not sampled in Smith *et al.* (2011), key taxa imperative to the phylogenetic structure within Bivalvia. Furthermore, a recent analysis using full mitochondrial genomes recovered the diphyly of Bivalvia, by virtue of *Solemya velum* nesting within Gastropoda (Plazzi *et al.* 2013). These results thus suggest that mitochondrial genome information fails to tease apart basal molluscan relationships among the eight classes and no robust phylogenetic signal for the monophyly of Bivalvia can be found (Plazzi *et al.* 2013). Discordance between mitochondrial and nuclear gene datasets is not a novelty as it has

been noted previously (Slade *et al.* 1994). Incongruence among datasets has been attributed to multiple characteristics within the mitochondrial genome; such discrepancies include degrees of recombination, modes of inheritance, incidence of introns, and in proportion of mitochondrial genome to haploid nuclear genome, where length of the mitochondrial genome is far less than length of the nuclear haploid genome (Sharma *et al.* 2012).

Discordant reconstructions have been recovered based on paleontological information, morphological information and molecular sequence data (Fig. 2.1), and these need to be resolved before continuing using bivalves as a preferred model to understand deep evolutionary patterns of paleoecology and biogeography. Empirical studies on patterning extinction and diversification as well as investigations on evolution of species ranges have relied on the bivalve fossil record (Valentine *et al.* 2006; Roy *et al.* 2009). Evaluations of large-scale biological diversity patterns, where there is a dramatic increase in the number of species and higher taxa from the poles to the tropics, was implicitly investigated using the first occurrences of bivalve genera in paleontological record (Jablonski *et al.* 2006). Recent analyses of diversification rates and divergence times for Protobranchia, demonstrate the signature of the end-Permian mass extinction in the phylogeny of extant protobranchs (Sharma *et al.* 2013). Bivalves are predominant macrofauna in the deep sea, where members of Anomalodesmata and Protobranchia persist and thrive. Bivalves have been used as model systems to investigate colonization, diversification, connectivity and evolutionary processes in the deep sea (Etter *et al.* 2005, 2011; Zardus *et al.* 2006; Stuart *et al.* 2008).

Here we provide the first phylogenomic reconstruction of Bivalvia toward resolving deep divergences within this group. All six major lineages of bivalves (Archiheterodonta, Euheterodonta [divided into Anomalodesmata and Inaequidonta *sensu* Bieler *et al.* in press],

Palaeoheterodonta, Protobranchia, and Pteriomorphia) were sampled resulting in 31 newly sequenced bivalve transcriptomes, in addition to six transcriptomes from representatives of all other molluscan lineages. This constitutes the most comprehensive phylogenomic dataset to date for inferring deep relationships within Bivalvia. Subsequent analyses obtained robust resolution of bivalve lineages, which corroborates traditional taxonomic relationships based on non-numerical cladistic analyses of paleontological and morphological data (Newell, 1965; Waller, 1990, 1998).

## Methods

### *Taxon Sampling*

Transcriptome data were obtained for 38 molluscan taxa, including 31 newly sequenced bivalve transcriptomes that had been selected to maximize the diversity of living bivalve lineages (Table 2.1). Full genome data were included for two taxa, *Lottia gigantea* (Simakov *et al.* 2013) (outgroup: Gastropoda) and *Pinctada fucata* (Takeuchi *et al.* 2012) (ingroup: Pteriomorpha). All six major bivalve lineages were represented with at least two species: Protobranchia (3), Pteriomorphia (6), Palaeoheterodonta (3), Archiheterodonta (3), Anomalodesmata (2) and Inaequidonta (17).

Tissues were preserved in three ways for RNA work: (1) flash-frozen in liquid nitrogen and immediately stored at -80 °C; (2) immersed in at least 10 volumes of RNAlater® (Ambion) and frozen at -80 °C or -20 °C; (3) transferred directly into TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and immediately stored at -80 °C.

**Table 2.1.** List of species sampled with voucher numbers and read pair information from subsequent data processing, included in the phylogenomic analyses. Taxonomy follows Bieler *et al.* (2013).

Taxa	Data Type	Voucher Number	Num of Raw reads (PE)	Post Filtering HIQual Reads	Assembled Contigs	Average Contig length	N50	Longest Contig	SwissProt Hits
<b>PROTOBRANCHIA</b>									
<i>Ennucula tenuis</i> (Montagu, 1808)	illumina PE	SRX091980/SRR331123	38,774,175	28,450,918	172,251	785	1,289	28,747	31,770
<i>Solenula velum</i> Say, 1822	illumina PE	SRX091478/SRR330465	33,298,527	22,761,869	98,156	1,215	2,646	32,991	24,520
<i>Yoldia limatula</i> (Say, 1831)	illumina PE	BivATol 19.1a/3a	40,216,909	26,199,671	15,867	533	521	13,885	5,950
<b>PTERIONOMORPHA</b>									
<i>Arca noae</i> Linnaeus, 1758	illumina PE	BivATol 116.1a	47,685,042	40,199,838	116,424	437	471	14,750	19,357
<i>Neocardia</i> sp.	illumina PE	DNA 106929	10,719,028	6,970,286	69,366	380	388	8,516	10,088
<i>Atrina rigida</i> (Lightfoot, 1786)	illumina PE	BivATol 14.1a	21,160,806	20,116,658	85,272	644	927	21,831	16,537
<i>Mytilus edulis</i> Linnaeus, 1758	illumina PE	Nahant 2011	25,644,478	20,427,253	80,287	612	642	19,256	27,737
<i>Pinctada fucata</i> (Gould, 1850)	Predicted Peptides	DRX001100/DRR001602	--	--	--	--	--	--	21,002
<i>Placopecten magellanicus</i> (Gmelin, 1791)	illumina PE	BivATol 360.1a	15,991,338	13,721,030	30877	758	913	30,116	16,354
<b>PALAEOTHERODONTA</b>									
<i>Lampsilis cardium</i> Rafinesque, 1820	illumina PE	BivATol 421.5a	19,317,443	14,819,846	108,039	493	589	13,298	13,494
<i>Margaritifera margaritifera</i> (Linnaeus, 1758)	illumina PE	BivATol 299.2d	15,172,806	10,596,660	50,736	514	509	13,399	8,158
<i>Neotrigonia margaritacea</i> (Lamarck, 1804)	illumina PE	DNA 106842	24,061,675	22,048,954	162,657	490	549	29,210	18,261
<b>ARCHIHERODONTA</b>									
<i>Astarte sulcata</i> (da Costa, 1778)	illumina PE	MCZ Sweden	37,671,660	32,670,541	76,320	451	512	6,976	9,586
<i>Eucassatella cumingii</i> (A. Adams, 1854)	illumina PE	BivATol 83.1b	42,454,880	21,838,500	102,094	395	395	13,256	10,051
<i>Cardites antiquata</i> (Linnaeus, 1758)	illumina PE	MCZ Spain	29,403,785	25,712,910	113,906	487	567	12,478	16,237
<b>ANOMALODESMATA</b>									
<i>Lyonsia floridana</i> Conrad, 1849	illumina PE	BivATol 248.1a	23,679,875	20,343,583	92076	588	838	8,775	25,319
<i>Myochama anomioides</i> Stutchbury, 1830	illumina PE	BivATol 84.1a	38,532,955	32,300,919	120,487	416	440	16,127	12,073
<b>INAEQUIDONTA</b>									
<i>Arctica islandica</i> (Linnaeus, 1767)	illumina PE	BivATol 191.3a	51,105,770	49,579,535	161,090	529	669	21,022	22,478
<i>Cerastoderma edule</i> (Linnaeus, 1758)	illumina PE	BivATol 21.1a	44,411,723	32,707,689	31,719	389	401	4,604	5,294
<i>Corbicula fluminea</i> (O.F. Müller, 1774)	illumina PE	BivATol 242.1a	51,063,111	48,293,353	176,007	576	763	27,648	30,161
<i>Cyrenoida floridana</i> Dall, 1896	illumina PE	BivATol 27.1a/2a	31,566,664	26,193,865	69,160	396	420	10,756	7,360
<i>Galeomma turtoni</i> [Anonymous], 1825	illumina PE	MCZ Spain	23,539,466	14,494,707	92,358	475	548	12,194	23,822
<i>Lasaea adansonii</i> (Gmelin, 1791)	illumina PE	BivATol 268.4	17,348,235	13,598,439	210,853	336	328	9,602	4,957
<i>Lamyachena hians</i> (Gmelin, 1791)	illumina PE	BivATol 289.1b	39,018,941	37,399,760	67,366	392	400	7,972	8,670
<i>Glossus humanus</i> (Linnaeus, 1758)	illumina PE	BivATol 200.1a	15,219,090	10,145,130	76,149	429	458	8,949	11,772
<i>Hiatella arctica</i> (Linnaeus, 1767)	illumina PE	BivATol 195.1a	37,170,745	36,448,617	73,557	487	576	13,375	15,063
<i>Phacoides pectinata</i> (Gmelin, 1791)	illumina PE	BivATol 278.1b	7,867,647	5,724,985	85,866	414	432	12,666	6,589
<i>Dipladonta</i> sp.	illumina PE	MCZ Panama	16,308,079	15,607,921	104,958	420	444	14,966	14,298
<i>Cycladicama cumingi</i> (Hanley, 1844)	illumina PE	BivATol 371.2c	18,444,567	17,256,202	77,024	438	484	7,676	10,419
<i>Rangia cuneata</i> (Sowerby I, 1832)	illumina PE	MCZ North Carolina	50,172,505	44,017,251	123,646	539	679	12,358	14,923
<i>Donacilla cornica</i> (Poli, 1791)	illumina PE	BivATol 406.1a	13,777,315	12,023,230	54,705	475	564	9,281	8,184
<i>Mya arenaria</i> Linnaeus, 1758	illumina PE	MCZ North Carolina	25,979,722	17,884,175	98,870	617	873	35,612	22,682
<i>Sphaerium corneum</i> (Linnaeus, 1758)	illumina PE	BivATol 194.1a	51,464,882	38,431,045	228,353	376	386	15,741	23,452
<i>Mercenaria campechiensis</i> (Gmelin, 1791)	illumina PE	MCZ North Carolina	17,562,216	16,027,302	54,108	460	520	11,917	6,947
<b>OUTGROUPS</b>									
<b>NEOMENIOMORPHA</b>									
<i>Greenland neomeniomorph</i> Pelseneer, 1906	illumina PE	SRX092156/SRR331902	35,228,112	24,109,577	69154	783	1008	28,982	58,972
<b>CEPHALOPODA</b>									
<i>Octopus vulgaris</i> Cuvier, 1797	illumina PE	SRA044948/SRS257947	8,250,668	5,009,720	63,005	711	826	12,825	19,977
<b>GASTROPODA</b>									
<i>Lottia gigantea</i> G. B. Sowerby I, 1834	Predicted Peptides	JGI	--	--	--	--	--	--	157,638
<b>SCAPHOPODA</b>									
<i>Gadilla talmiei</i> (Dall, 1897)	illumina PE	SRX092154/SRR331897	37,971,066	37,024,753	106,509	459	522	13,484	29,462
<b>POLYPLACOPHORA</b>									
<i>Chiton olivaceus</i> Spengler, 1797	illumina PE	SRA061738	23,189,291	17,395,660	63,253	601	644	11,858	29,067
<b>MONOPLACOPHORA</b>									
<i>Laevipilina hyalina</i> J. H. McLean, 1979	illumina PE	SRX091470/SRR330425	29,983,919	21,400,869	184,307	563	714	29,374	49,370

### *RNA Isolation*

Tissue excisions were always performed with sterilized razor blades rinsed in RNaseZap® (Ambion, Texas, US). All cleaning procedures were operated as quickly as possible to avoid RNA degeneration in an RNase-free and cold environment using liquid nitrogen.

### *mRNA extraction*

Total RNA was isolated from tissues preserved as described above. Total RNA extraction followed by mRNA purification for following published protocols (Regier *et al.* 2005). Following mRNA purification, samples were treated with Ambion® TURBO DNA-free™ DNase following manufacturer's protocol to remove residual genomic and rRNA contaminants.

Quantity and quality (purity and integrity) of mRNA were assessed by three different methods. We measured the absorbance at different wavelengths using a NanoDrop ND-1000 UV spectrophotometer (Thermo Fisher Scientific, Wilmington, Massachusetts, USA). Quantity of mRNA was also assessed with the fluorometric quantitation performed by the Qubit® Fluorometer (Invitrogen, California, USA). Also, capillary electrophoresis in an RNA Pico 6000 chip was performed using an Agilent Bioanalyzer 2100 System with the “mRNA pico Series II” assay (Agilent Technologies, California, USA). Integrity of mRNA was estimated by the electropherogram profile and lack of rRNA contamination (based on rRNA peaks for 18S and 28S rRNA given by the Bioanalyzer software).

### *Next-Generation Sequencing (NGS)*

NGS was carried out using the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, California, USA) at the FAS Center for Systems Biology at Harvard University. Protocols used

for subsequent cDNA synthesis from extracted mRNA were modified from Regier *et al.* (2005), where SuperScript® III Reverse Transcriptase (RT) was used to amplify cDNA gene products. cDNA was ligated to Illumina TruSeq RNA multiplex adaptor sequences using the TruSeq RNA Sample Prep Kit (Illumina). No more than 6 adaptors were used per individual multiplexed sequencing run. Size-selected cDNA fragments of 250-350 bp excised from a 2% agarose gel were amplified using Illumina PCR Primers for Paired-End reads (Illumina) and 18 cycles of the PCR program 98 °C-30 s, 98 °C-10 s, 65 °C-30 s, 72 °C-30 s, followed by an extension step of 5 min at 72 °C.

The concentration of the cDNA libraries was measured with the QubiT® dsDNA High Sensitivity (HS) Assay Kit using the QubiT® Fluoremeter (Invitrogen, Carlsbad, California, USA). The quality of the library and size selection were checked using the "HS DNA assay" in a DNA chip for Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). cDNA libraries were considered successful when the final concentration was higher than 1 ng/μL and the Bioanalyzer profile was consistent with prior size selected cDNA fragmentation ranges. Successful libraries were sequenced using normalized concentrations of 10 nM or 7nM. Concentrations of sequencing runs were normalized based on final concentrations of fragmented cDNA. Illumina sequenced paired-end reads were 101 bps.

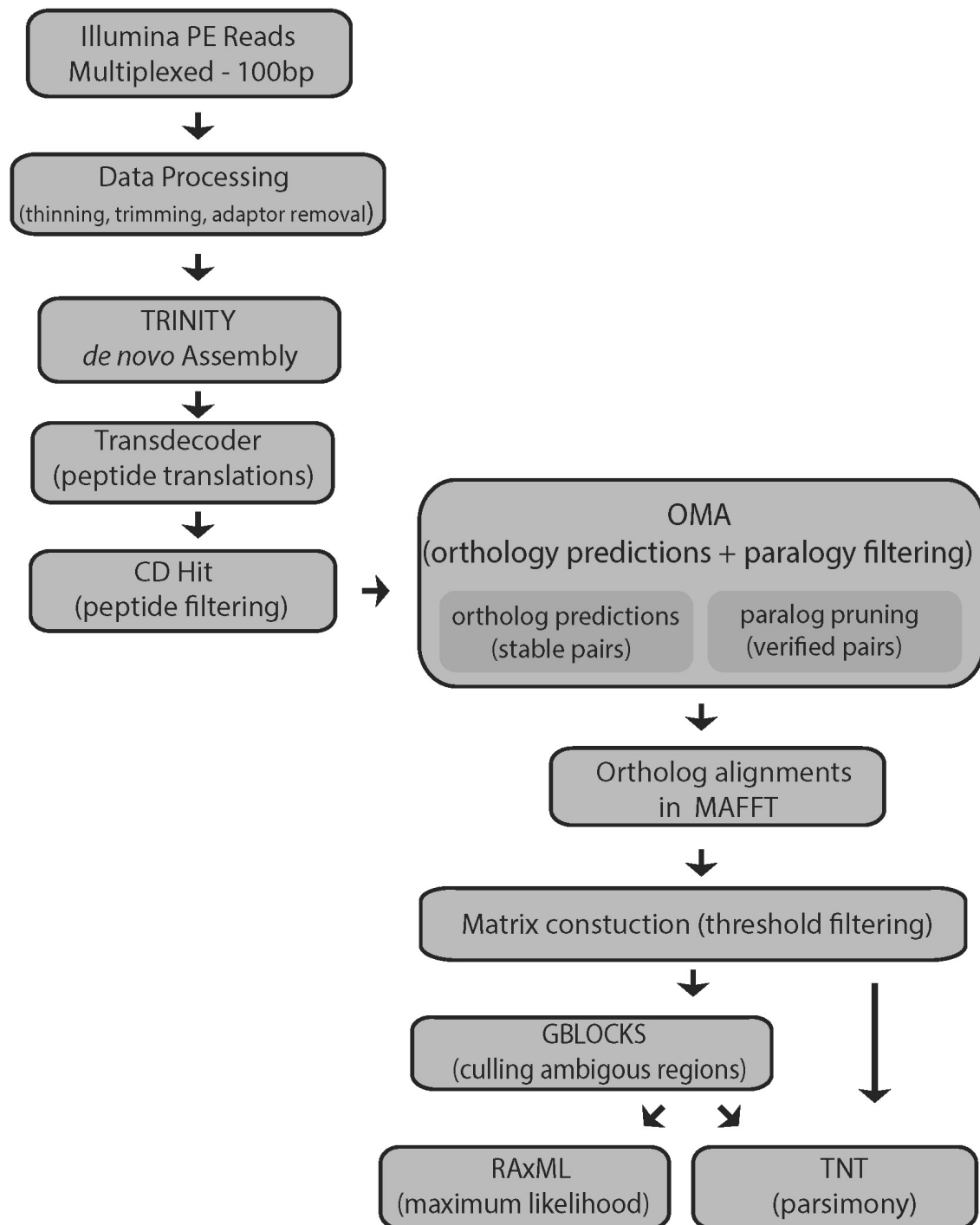
**Bioinformatic pipeline** – including: data processing; *de novo* assembly; orthology assignment and matrix construction; and phylogenetic analysis – is outlined in Fig. 2.2.

### *Data processing*

Illumina HiSeq 2000 pair-end reads obtained per taxon ranged from 7 867 647 to 51 464 822 per taxon (Table 2.1). Data (unprocessed reads) obtained from the Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) were downloaded as raw reads and processed in the same manner as the newly generated transcriptome data. Quality of reads was visualized with FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). Initial removal of low quality reads and TruSeq multiplex index adaptor sequences (Illumina) was performed with Trim Galore! v. 0.3.1 ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)), setting the quality threshold to minimum Phred score of 30. Illumina TruSeq multiplex adaptor sequences were trimmed, specific to the adaptor used in sequencing with the paired-end data flag. A second round of quality threshold filtering (minimum Phred 35) as well as removal of rRNA sequence contamination was conducted in Agalma v.0.3.2 using the “pre-assemble” pipeline (Smith *et al.* 2012). The “pre-assemble” pipeline filters rRNA sequences, after first randomizing input sequences for both pairs of reads, creating a subassembly and annotates rRNA sequences based on a subassembly of the data, then remove clusters in which one or both reads map to rRNA sequences.

### *De novo Assembly*

Quality filtered and sanitized high quality reads (Table 2.1) were assembled with the Trinity *de novo* Assembler (release 2011-07-13) with 100g of job memory and a path reinforcement distance of 50. The number of contigs, the mean contig length, the N50, and the maximum contig length were reported for each *de novo* assembly (Table 2.1). Contigs were mapped against the Swissprot database using the blastx program of the BLAST suite and the number of contigs



**Figure 2.2.** Bioinformatics pipeline, including: data processing; *de novo* assembly; orthology assignment and matrix construction; and phylogenetic analysis.



returning blast hits were quantified (Table 2.1). Nucleotide sequences were translated with Transdecoder using default parameters (Grabherr *et al.* 2011). Subsequent peptide translations were filtered for redundancy and uniqueness using CD-Hit v.4.6.1 under default parameters, and a 95% similarity threshold (Fu *et al.* 2012). Genome data from *Lottia gigantea* and *Pinctada fucata* were incorporated using predicted peptide sequences obtained from public sources.

#### *Orthology assignment and matrix construction*

Orthology assessment was conducted using OMA standalone v.0.99t (Roth *et al.* 2008), on 64 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu), using default parameters, except with a minimum alignment score of 200, Length tolerance ratio of 0.75, and a minimum sequence length of 100. A total of 68 828 parsimony informative putative orthogroups (>4 taxa) were obtained; from here on orthogroups and genes are referred to interchangeably. Resultant gene clusters were aligned with MAFFT (Kato & Toh 2008) prior to concatenation.

We constructed six phylogenetic matrices from the translated amino acid sequences. Three supermatrices were constructed based on gene occupancy threshold filters—meaning that a gene was selected if found in more than or equal to the established threshold; a 50% threshold would select all genes present in 50% or more of the included taxa. The 37.5, 50, and 75 percent gene occupancy matrices were then trimmed with Gblocks (Castresana 2000) to cull regions of dubious alignment, to generate three more data matrices to be used in downstream phylogenetic reconstructions.

### *Phylogenetic analysis*

Two optimality criteria were employed to reconstruct bivalve relationships, maximum likelihood and equal weights parsimony, the latter as a fast but simplistic method, as no amino acid transformation step matrices are readily available for parsimony analyses. Maximum likelihood tree searches on the three data matrices treated with Gblocks were conducted with RAxML version 7.2.7 (Stamatakis 2006). Concomitantly, tree searches were conducted for all 6 data matrices (3 treated with Gblocks and 3 untreated matrices) under equal weights parsimony in TNT - Tree analysis using New Technology (Goloboff *et al.* 2008).

Maximum likelihood analyses in RAxML specified a protein model of sequence evolution with corrections for a discrete gamma distribution with the Le and Gascuel (LG) model (Le & Gascuel 2000) to conduct the tree searches, with 100 independent replicates. Bootstrap resampling was conducted for 1000 replicates specifying a protein model of sequence evolution with corrections for a discrete gamma distribution using the WAG model (Wheeler & Goldman 2001) and were thereafter mapped onto the optimal tree from the independent searches. The three untreated matrices were too large to analyze with these tools.

TNT searches of each of the 6 matrices were conducted using 100 replications, 10 rounds of tree fusing (Goloboff 1999) and ratcheting (Nixon 1999). Bootstrap resampling consisted of 1000 replications; resampling frequencies of recovered clades were summarized on the optimal parsimony or strict consensus of most-parsimonious trees.

## Results

### *Supermatrices*

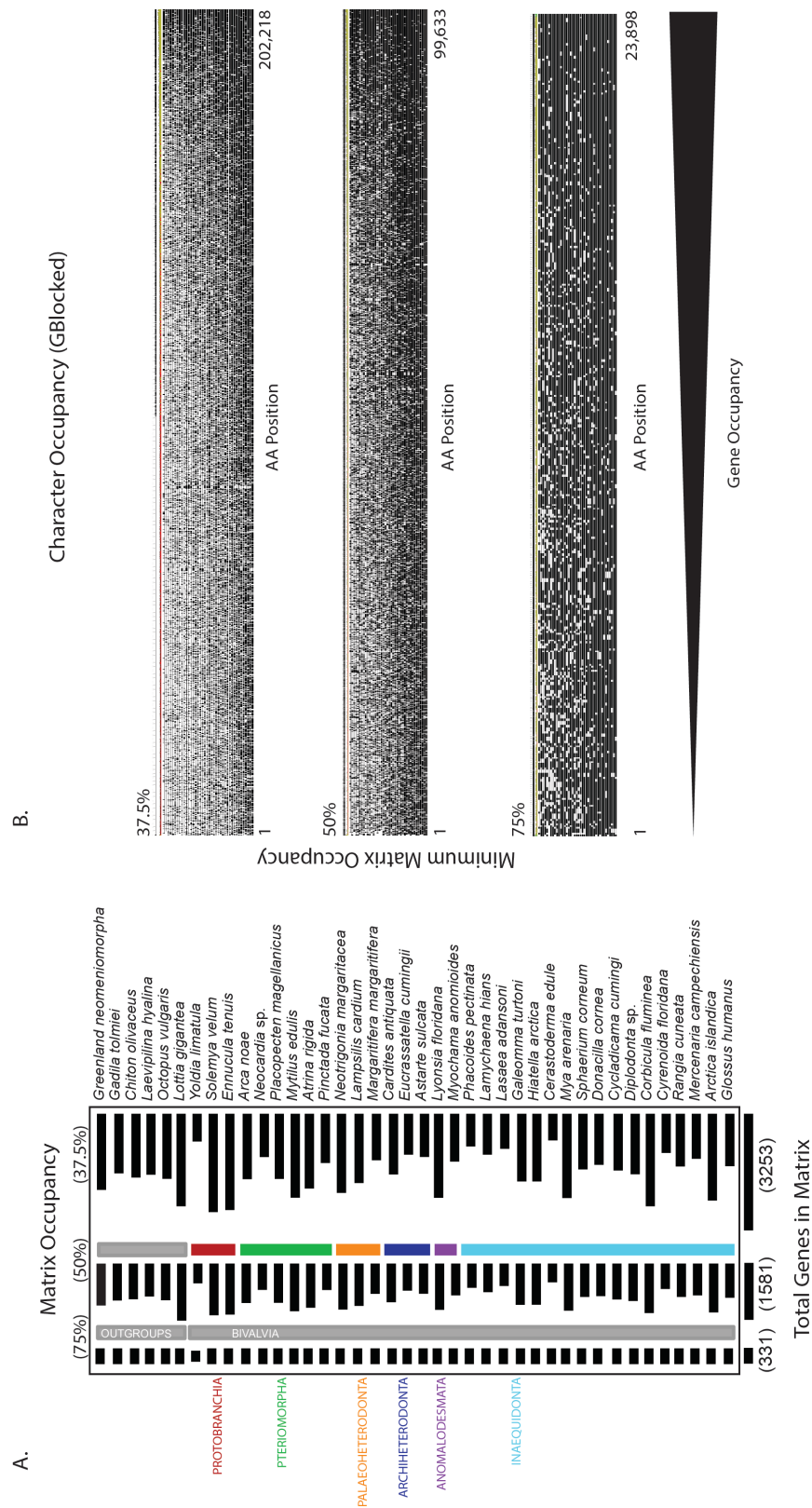
Concatenated matrices were compiled using percent gene occupancy, and concomitantly, either treated to cull regions of dubious (or with a substantial amount of missing information) homology in the alignment, or left untreated. The largest matrix with 3 253 genes was compiled with a 37.5 percent gene occupancy threshold ( $\leq 15$  taxa) for each cluster. The 37.5 percent gene occupancy matrix resulted in 1 609 099 aligned amino acid (AA) sites. The intermediate matrix was compiled with 50 percent gene occupancy ( $\leq 20$  taxa) and resulted in 649 533 aligned AA sites from 1 581 genes. The smallest matrix, with the densest percent gene occupancy of 75 percent ( $\leq 30$  taxa), consisted of 331 genes, corresponding to 104 135 aligned AA sites. The number of genes present in the matrices varied by taxon, with the most genes being represented by two protobranch taxa, *Ennucula tenuis* and *Solemya velum* (Fig. 2.3a). All six matrices contain data for all of the 40 species included in the study, though taxa varied in gene representation (Table 2.2). Terminals with the least amount of total parsed characters, were *Cerastoderma edule* and *Yoldia limatula*, with only 22.78% and 23.7% of the total genes present in the largest matrix (37.5%, 7 998 genes) (Table 2.2).

Untreated datasets resulted in larger matrices, with respect to character count, compared to those of the treated datasets. The treated datasets resulted in matrices of 202 218 aligned sites (37.5% occupancy matrix); 99 633 aligned sites (50% occupancy matrix); 23 898 aligned sites (75% occupancy matrix) aligned amino acid sites, for the three respectively. Character occupancy was slightly higher than the gene occupancy threshold for all treated datasets (e.g. the

50% gene occupancy treated with Gblocks resulted in 65% character occupancy for the respective dataset) (Table 2.3, Fig. 2.3b).

**Table 2.2.** List of number and ratio of genes sampled per taxa, for each minimum gene occupancy threshold used for super-matrix construction: 37.5%, 50% and 75 %.

Species	MATRIX OCCUPANCY (NUMBER OF GENES)					
	37.5 % (3253)	Total (%)	50 % (1581)	Total (%)	75% (331)	Total (%)
<b>PROTOBRANCHIA</b>	---	---	---	---	---	---
<i>Ennucula tenuis</i> (Montagu, 1808)	2683	82.48%	1414	89.44%	314	94.86%
<i>Solemya velum</i> Say, 1822	2741	84.26%	1432	90.58%	319	96.37%
<i>Yoldia limatula</i> (Say, 1831)	771	23.70%	545	34.47%	228	68.88%
<b>PTERIOMORPHA</b>	---	---	---	---	---	---
<i>Arca noae</i> Linnaeus, 1758	1823	56.04%	1092	69.07%	290	87.61%
<i>Neocardia</i> sp. Bernardi, 1897	1208	37.13%	728	46.05%	226	68.28%
<i>Atrina rigida</i> (Lightfoot, 1786)	2085	64.09%	1222	77.29%	310	93.66%
<i>Mytilus edulis</i> Linnaeus, 1758	2339	71.90%	1321	83.55%	305	92.15%
<i>Pinctada fucata</i> (Gould, 1850)	1371	42.15%	731	46.24%	169	51.06%
<i>Placopecten magellanicus</i> (Gmelin, 1791)	1817	55.86%	1085	68.63%	290	87.61%
<b>PALAEOTHERODONTA</b>	---	---	---	---	---	---
<i>Lampsilis cardium</i> Rafinesque, 1820	1932	59.39%	1171	74.07%	311	93.96%
<i>Margaritifera margaritifera</i> (Linnaeus, 1758)	1297	39.87%	839	53.07%	262	79.15%
<i>Neotrigonia margaritacea</i> (Lamarck, 1804)	2204	67.75%	1275	80.65%	315	95.17%
<b>ARCHIOTHERODONTA</b>	---	---	---	---	---	---
<i>Astarte sulcata</i> (da Costa, 1778)	1223	37.60%	835	52.81%	260	78.55%
<i>Eucassatella cumingii</i> (A. Adams, 1854)	1156	35.54%	748	47.31%	247	74.62%
<i>Cardites antiquata</i> (Linnaeus, 1758)	1722	52.94%	1071	67.74%	287	86.71%
<b>ANOMALODESMATA</b>	---	---	---	---	---	---
<i>Lyonsia floridana</i> Conrad, 1849	2345	72.09%	1285	81.28%	309	93.35%
<i>Myochama anomioidea</i> Stutchbury, 1830	1335	41.04%	881	55.72%	269	81.27%
<b>INAEQUIDONTA</b>	---	---	---	---	---	---
<i>Arctica islandica</i> (Linnaeus, 1767)	2419	74.36%	1353	85.58%	314	94.86%
<i>Cerastoderma edule</i> (Linnaeus, 1758)	741	22.78%	518	32.76%	211	63.75%
<i>Corbicula fluminea</i> (O.F. Müller, 1774)	2580	79.31%	1374	86.91%	300	90.63%
<i>Cyrenoida floridana</i> Dall, 1896	1093	33.60%	701	44.34%	238	71.90%
<i>Galeomma turtoni</i> [Anonymous], 1825	1880	57.79%	1145	72.42%	306	92.45%
<i>Lasaea adansonii</i> (Gmelin, 1791)	967	29.73%	619	39.15%	227	68.58%
<i>Lamychaena hians</i> (Gmelin, 1791)	1151	35.38%	790	49.97%	272	82.18%
<i>Glossus humanus</i> (Linnaeus, 1758)	1463	44.97%	946	59.84%	282	85.20%
<i>Hiatella arctica</i> (Linnaeus, 1767)	1905	58.56%	1141	72.17%	292	88.22%
<i>Phacoides pectinata</i> (Gmelin, 1791)	928	28.53%	671	42.44%	249	75.23%
<i>Diplodonta</i> sp. Bronn, 1831	1684	51.77%	1037	65.59%	301	90.94%
<i>Cycladicama cumingi</i> (Hanley, 1844)	1581	48.60%	995	62.93%	287	86.71%
<i>Rangia cuneata</i> (Sowerby I, 1832)	1469	45.16%	925	58.51%	265	80.06%
<i>Donacilla cornea</i> (Poli, 1791)	1422	43.71%	903	57.12%	282	85.20%
<i>Mya arenaria</i> Linnaeus, 1758	2348	72.18%	1306	82.61%	317	95.77%
<i>Sphaerium corneum</i> (Linnaeus, 1758)	1552	47.71%	923	58.38%	260	78.55%
<i>Mercenaria campechiensis</i> (Gmelin, 1791)	1253	38.52%	881	55.72%	284	85.80%
<b>OUTGROUPS</b>	---	---	---	---	---	---
<b>NEOMENIOMORPHA</b>	---	---	---	---	---	---
<i>Greenland neomeniomorph</i> Pelseneer, 1906	2121	65.20%	1163	73.56%	270	81.57%
<b>CEPHALOPODA</b>	---	---	---	---	---	---
<i>Octopus vulgaris</i> Cuvier, 1797	1812	55.70%	1015	64.20%	251	75.83%
<b>GASTROPODA</b>	---	---	---	---	---	---
<i>Lottia gigantea</i> G. B. Sowerby I, 1834	2580	79.31%	1354	85.64%	301	90.94%
<b>SCAPHOPODA</b>	---	---	---	---	---	---
<i>Gadila tolmiei</i> (Dall, 1897)	1658	50.97%	1022	64.64%	254	76.74%
<b>POLYPLACOPHORA</b>	---	---	---	---	---	---
<i>Chiton olivaceus</i> Spengler, 1797	1779	54.69%	987	62.43%	241	72.81%
<b>MONOPLACOPHORA</b>	---	---	---	---	---	---
<i>Laevipilina hyalina</i> J. H. McLean, 1979	1699	52.23%	913	57.75%	229	69.18%



**Figure 2.3.** (A) Genes sampled per taxon per percent occupancy threshold. Size of bar is relative to number of genes present in each super matrix per taxon. (B) Character occupancy for three super-matrices treated with Gblocks. For each particular site, black indicated presence of an amino acid and white indicates an absence of amino acid. Genes sampled are ordered minimum matrix occupancy per gene, to maximum matrix occupancy per gene.

**Table 2.3.** Comparisons of all nine constructed super-matrices based on data partition size, prior and subsequent to treatment for length variability and summary statistics. Bootstrap values shown for all nine analyses for each corresponding matrix using equal weights parsimony analyses in TNT, with 100 independent starts and 1000 bootstrap replicates.

	Matrix Occupancy					
	37.5 % (3253 genes)		50 % (1581 genes)		75 % (331 genes)	
	Untreated	Gblocked	Untreated	Gblocked	Untreated	Gblocked
Alignment Size	1,609,099	202,218	649,533	99,633	104,135	23,898
Percent Missing Data	71.16%	46.97%	60.38%	35.16%	40.40%	16.97%
Average Ungapped length	464,031	107,232	257,322	64,603	62,101	17,258
Standard Deviation	229,816	31,783	96,813	14,786	12,193	1,936
Maximum Ungapped length	1,069,299	169,364	465,861	90,101	78,754	19,647
Maximum Ungapped Length	127,899	50,291	91,535	36,008	34,277	10,573
Monophyly of (BS)	--	--	--	--	--	--
Bivalvia	100	100	100	100	100	45
Heteroconchia	100	99	98	80	87	--
Heterodonta	100	100	100	97	98	78
Protobranchia	93	97	91	--	23	--
Pteriomorpha	100	100	100	100	100	100
Paleoheterodonta	100	100	100	100	100	100
Archiheterodonta	100	100	100	100	100	100
Anomalodesmata	100	100	100	100	100	100
Inequidonta	100	100	100	100	100	100

#### *Phylogenetic analyses – maximum likelihood (ML)*

RAxML resulted in tree topologies with a  $\ln L = -3\,349\,188.52$ ,  $\ln L = -1\,807\,910.03$ , and  $\ln L = 30\,479.037$  for the Gblocked datasets of 37.5, 50, and 75 percent gene occupancy matrices respectively (Fig. 2.4). ML tree topologies of each data set recovered highly congruent topologies throughout Bivalvia. All but one dataset recovered the monophyly of all six major lineages with high support (BS = 100), with the exception of Protobranchia. Protobranchia was recovered in all but one analyses (75% gene occupancy matrix), in which *Yoldia limatula* falls outside of the clade sister to the remaining Autobranchia, while in the 50% gene occupancy matrix analysis it recovered monophyly of Protobranchia with low support (BD=57).

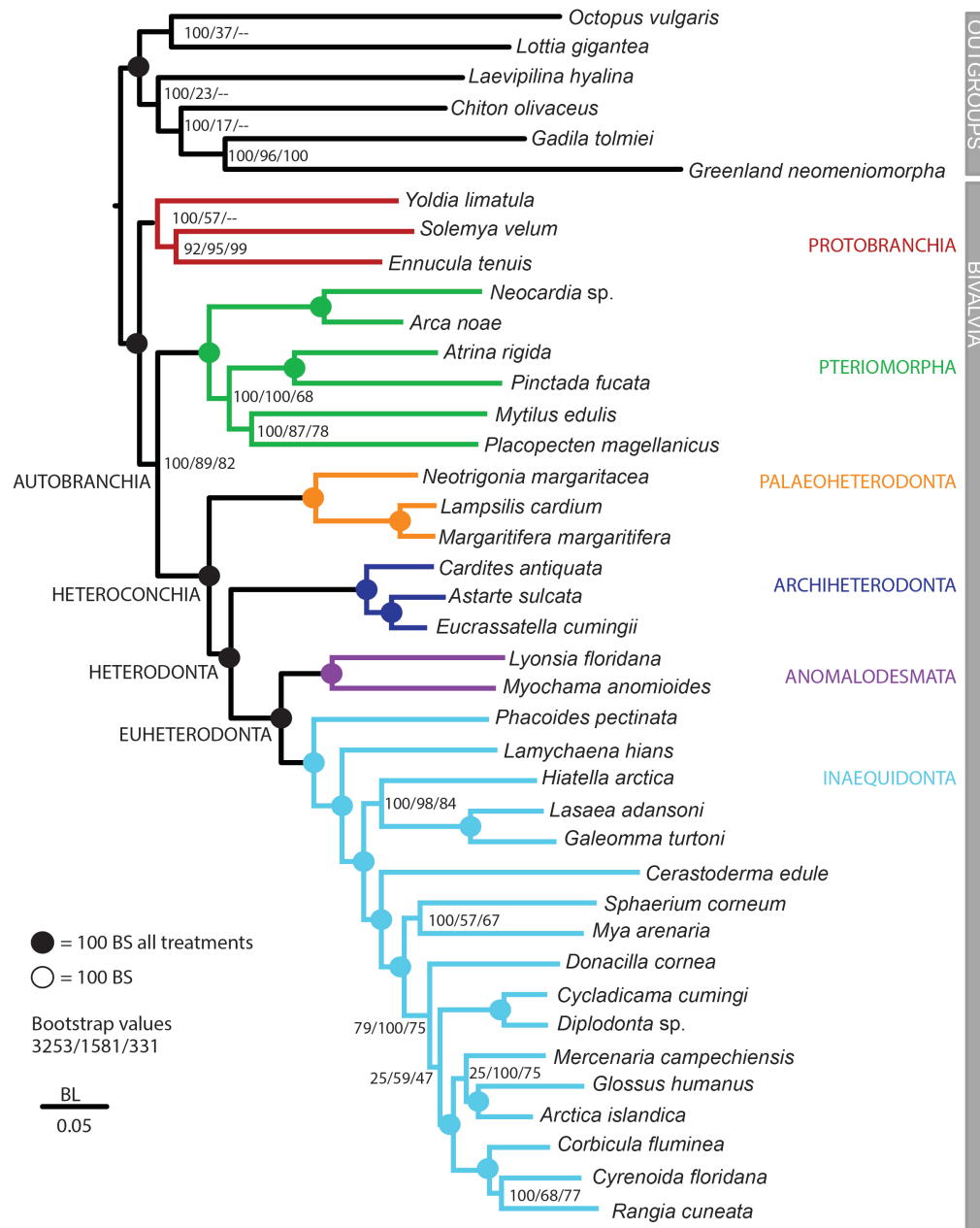
The smallest dataset (75% gene occupancy; 331 genes) was topologically incongruent with respect to relationships within the outgroup taxa, however monophyly of Bivalvia was recovered in all analyses with high support (BS=100). Monophyly was recovered with high

support (BS=100) for most higher-level bivalve relationships: Heteroconchia, Heterodonta and Euheterodonta. Autobranchia was recovered in all datasets, though moderately supported in two analyses: 50% gene occupancy (BS = 89) and 75% gene occupancy (BS=82) and highly supported in the 37.5% gene occupancy matrix (BS = 100).

All three analyses recovered highly congruent and highly supported interfamilial relationships throughout, with the exception of the placement of *Yoldia limatula* in Protobranchia—this species is one of the poorest represented in all the matrices (Table 2.2). Though all tree topologies were congruent in the three analyses among Euheterodonta, several relationships were not robustly supported in all analyses (Fig. 2.4).

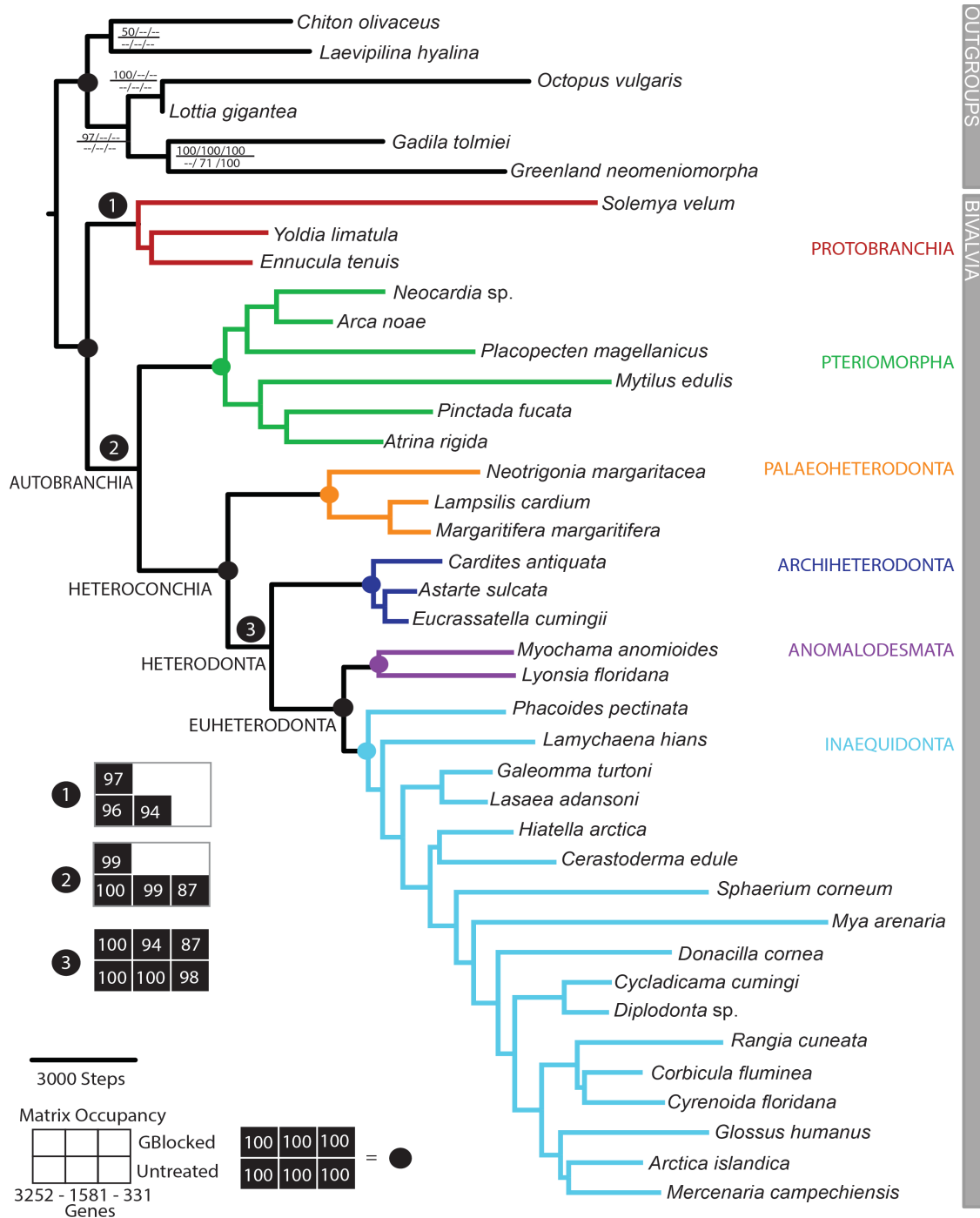
#### *Phylogenetic analyses – equal weights parsimony (TNT)*

All major bivalve lineages were recovered as monophyletic in all analyses with the exception of the Protobranchia and the subsequent mutual monophyly of Autobranchia (Fig. 2.5). Higher-level taxonomic groups were also recovered in a majority of the analyses with high support. All reconstructions made with both Gblocked and untreated datasets for the largest matrix (37.5% gene occupancy; 3253 genes) recovered monophyly of Protobranchia (BS = 97; BS = 96), and Autobranchia (BS = 99; BS = 100). All analyses recovered Bivalvia, Heteroconchia and Euheterodonta with maximum nodal support for all matrices (BS = 100). Heterodonta was recovered as monophyletic with high support in all analyses (BS = 100-87). The smallest datasets (331 genes), both untreated and treated, favored the non-monophyly of Protobranchia, where the treated dataset also favored the mutual non-monophyly of Autobranchia, as *Yoldia limatula* nested within this clade. For the 50% gene occupancy matrix, analyses for the treated and untreated datasets were congruent with the exception of the mutual monophyly of Protobranchia



**Figure 2.4.** Phylogenetic relationships of Archiheterodonta based on maximum likelihood analysis of three treated super-matrices. Numbers on nodes indicate bootstrap resampling frequencies. Colors in tree topology correspond to the six major lineages (red: Protobranchia; green: Pteriomorpha; orange: Palaeoheterodonta; indigo: Archiheterodonta; purple: Anomalodesmata; light blue: Inaequidonta).





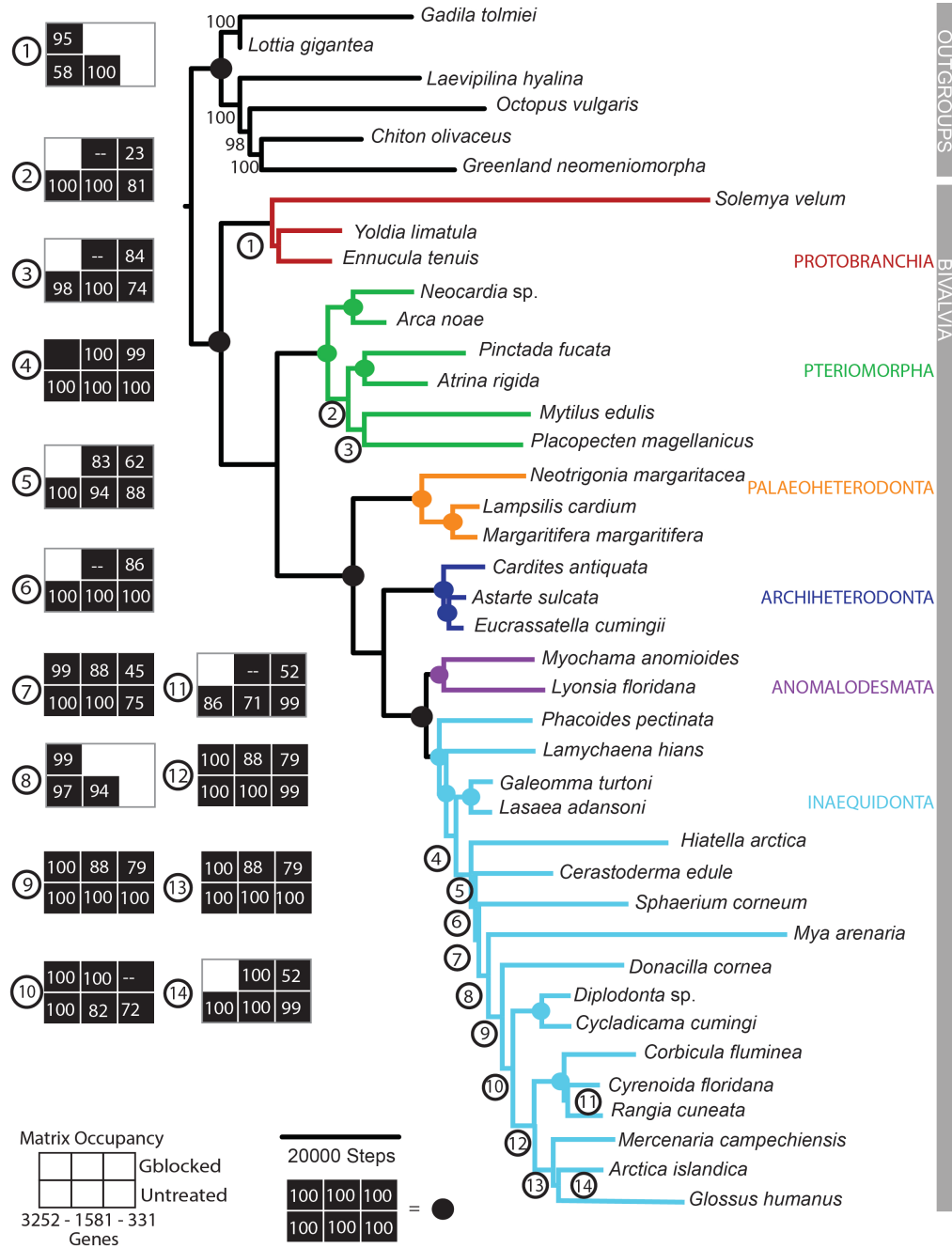
**Figure 2.5.** Phylogenetic relationships of major lineages of Bivalvia based on equal weights parsimony in TNT. Navajo rugs correspond to three nodes of interest. Colors in Navajo rugs correspond to each dataset; numbers in Navajo rugs indicate bootstrap resampling frequencies from treated dataset analyses (top row) or the untreated dataset (bottom row). Nodes with 100 Bootstrap support in all analyses are indicated by a filled circle. Failure to retrieve a node is indicated as a white entry (without a number indicating nodal support). Colors in tree correspond to the six bivalve lineages (as in Figure 2.3).

and Autobranchia which was recovered with high support (BS = 94; BS = 99, respectively) in only the untreated dataset.

Matrix occupancy and culling for areas of ambiguity in the alignment produced differing interfamilial tree topologies (Fig. 2.6). Relationships within Protobranchia of the relationships of Nuculida + Nuculanida sister to *Solemya* was contingent on the recovery of the monophyly of the clade, if Protobranchia was recovered, this relationship was always found. Internal relationships of Pteriomorphia were congruent (Fig. 2.6), with the exception of the analyses using the largest treated matrix (37.5% gene occupancy – Fig. 2.5). *Arca noae* was sister to *Neocardia* sp. in all analyses (BS = 100) and likewise, *Atrina rigida* was always recovered as related to *Pictada fuctada* (BS = 100). *Mytilus edulis* and *Placopecten magellanicus* formed a clade sister to the *A. rigida* + *P. fuctada* clade in all analyses except the analyses on the treated 37.5% gene occupancy matrix, where *M. edulis* was recovered in the *A. noae* + *Neocardia* sp. clade, though this topology was not supported. Within Euheterodonta, basal relationships were stable and were recovered in all topologies (BS = 100), while derived relationships in Euheterodonta were not as consistent. The lucinid representative, *Phacoides pectinata*, was recovered at the base of Inaequidonta followed by a grade of *Lamychaena hians*, and Galeommatoidea (*Galeomma turtoni* + *Lasaea adansonii*) in all analyses (BS = 100).

## Discussion

Transcriptomic-scale analyses obtained robust resolution and stable relationships of bivalve lineages, corroborating most traditional relationships based on non-numerical cladistic analyses of paleontological and morphological data (e.g., Newell 1965; Waller 1990, 1998) and many

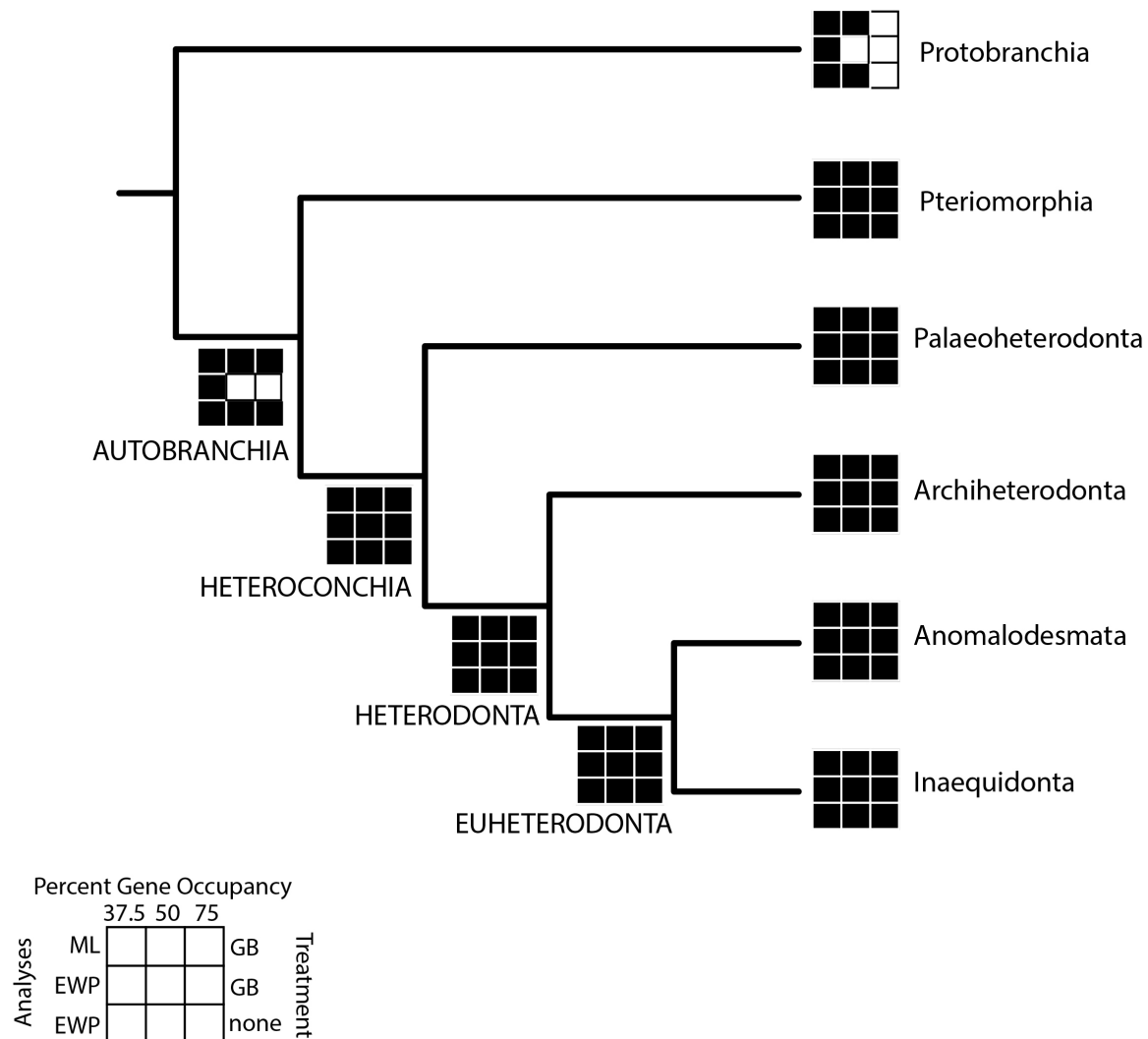


**Figure 2.6.** Inter-familial phylogenetic relationships of Bivalvia based on equal weights parsimony inference of nine analyses. Tree topology shown is for the untreated 37.5% gene occupancy matrix. Numbers in Navajo rugs indicate bootstrap resampling frequencies from treated dataset analyses (top row) or the untreated dataset (bottom row). Failure to retrieve a node is indicated as a white entry (without a number indicating nodal support). Nodes with 100 bootstrap support in all analyses are indicated by a filled circle on node. Colors in tree correspond to the six bivalve lineages (as in Figure 2.3).

recent phylogenetic analyses of bivalves. Supermatrices comprised between 20 452 (Gblocked, 331 gene regions) and up to 1 609 099 (untreated, 3253 gene regions) aligned AA sites for the smallest and largest datasets, respectively. This constitutes the most comprehensive phylogenomic dataset to date for inferring deep relationships within Bivalvia, and the largest molecular data set assembled for a group of animals in both, number of genes and number of characters. Robust support for higher-level taxonomic relationships was obtained in all analyses for Bivalvia, Autobranchia, Heteroconchia and Heterodonta (Fig. 2.7).

Monophyly of Protobranchia is supported in previous phylogenomic analyses (Kocot *et al.* 2011; Smith *et al.* 2011), and recent Sanger-based molecular analyses (Sharma *et al.* 2011; Bieler *et al.* in press). Tree topologies obtained in this study recovered incongruent relationships of Protobranchia among analyses based on different optimality criterion. It should be noted that probabilistic approaches used datasets which discarded highly variable regions, while equal weights parsimony included the same datasets without hypervariable regions as well as datasets comprised of all sequence information, but lack of appropriate transformation cost matrices for amino acids. Tree topologies recovered all iterations of relationships among the protobranch lineages, where parsimony analyses favored Solemyida sister to a clade of Nuculida + Nuculanida (as in Sharma *et al.* 2012, 2013, both studies based on maximum likelihood and Bayesian methodologies), maximum likelihood analyses recovered Nuculanida sister to Solemyida + Nuculida (as in e.g., Giribet & Wheeler 2002, based on parsimony). Both relationships have thus been hypothesized based on morphological and molecular analyses (Fig. 2.1) and using different methodologies for data analysis. Previously, studies on bivalve phylogenetics recovered Opponobranchia, including Nuculida + Solemyida, and a clade

consisting of Nuculida + Autobranchia (Giribet 2008). Here this relationship is recovered in one analysis based on the smallest treated dataset (75%, 331 genes), though not supported. Our results are thus not sufficient to resolve the phylogenetic relationships of Protobranchia, and we



**Figure 2.7.** Consensus of all none topologies obtained. Navajo rugs correspond each node of interest; Navajo rugs indicate recovery of monophyly in RAxML analyses of treated datasets (top row) or TNT analyses of treated datasets (middle row) and untreated datasets (bottom row). Black entries indicate support for monophyly; failure to retrieve a node is indicated as a white entry.

suspect that the low gene representation of *Yoldia limatula* (not generated for this study) and the sparse taxon sampling within Protobranchia (all obtained from the study of Smith *et al.* 2011) may be responsible for the lack of substantial improvement at the base of the bivalve tree. Further work is thus required to assess the final relationships of Protobranchia, where sampling should increase to mirror known diversity in this group (see Sharma *et al.* 2013).

Results for Autobranchia and its main constituent lineages (Pteriomorphia, Palaeoheterodonta, Archiheterodonta, Anomalodesmata and Inaequidonta) are however well resolved and supported in virtually all analyses (Figs. 2.4-6). A remaining contentious issue is however the position of Archiheterodonta with respect to Palaeoheterodonta or Euheterodonta. In contrast to recent phylogenetic analyses which favor the sister relationship of Archiheterodonta + Palaeoheterodonta and the subsequent non-monophyly of Heterodonta (Wilson *et al.* 2010; Sharma *et al.* 2012; Bieler *et al.* in press), the more traditional monophyly of Heterodonta is recovered in all analyses with robust support, closing this debate in the recent bivalve literature. This is not without some controversy, as the traditional Heterodonta hypothesis did not include Anomalodesmata (e.g., Waller 1990, 1998; Cope 1997), while virtually all modern studies, molecular or morphological, group Anomalodesmata with the non-archiheterodont heterodonts (i.e., Inaequidonta; e.g., Giribet & Wheeler 2002; Giribet & Distel 2003; Carter *et al.* 2011; Sharma *et al.* 2012; Bieler *et al.* in press; but see the mitochondrial papers of Plazzi & Passamonti 2010 and Plazzi *et al.* 2011). We thus corroborate these modern studies in finding monophyly of Euheterodonta, which divides into Anomalodesmata and Inaequidonta.

Euheterodonta was thus recovered in all analyses with strong support, as was the sister group relationship of Anomalodesmata and Inaequidonta. Internal relationships of Inaequidonta are for the first time well supported and congruent with respect to the basalmost lineages across

analyses. *Phacoides pectinata* (Lucinidae), a species characterized by chemosymbiotic sulphide-oxidizing bacteria housed in the ctenidial, was unambiguously recovered as the sister group to the remaining Inaequidonta (BS = 100), a relationship supported previously (e.g., Giribet & Distel 2003; Taylor *et al.* 2007; Wilson *et al.* 2010; Bieler *et al.* in press). Gastrochaenidae (*Lamychaena hians*), a group difficult to place phylogenetically in most previous studies (see a discussion in Bieler *et al.* in press), appears as the next branch, sister group to the remaining inaequidonts. Monophyly of Galeommatoidea (Galmeommatidae + Lasaeidae), raised in taxonomic rank to superfamilial status by Taylor *et al.* (2007), is also supported, and constitutes the sister group to the remaining inaequidonts. Neoheterodonte (sensu Taylor *et al.* 2007) was recovered as a highly supported clade. This previously unranked clade, contains a majority of inaequidont lineages, and is congruent with recent molecular analyses (Sharma *et al.* 2012; Bieler *et al.* in press). Some of the most derived lineages of Inaequidonta were not robustly supported, though Ungulinidae and a clade comprising Cyrenoididae + Corbiculidae was recovered as well supported in all analyses. Within the Cyrenoididae + Cordibulidae clade, a counterintuitive placement of *Rangia cuneata* (Mesodesmatidae) as the sister group to these lineages, renders Mactroidea (Mactridae + Mesodesmatidae) not monophyletic, while this clade is well supported in other molecular analyses (Taylor *et al.* 2007; Bieler *et al.* in press).

The relationships among the inaequidont families remain somehow controversial, but this study does not sample all families and was designed exclusively to test the deep divergences among the main lineages of bivalves. This phylogenomic approach resolves several controversial aspects of bivalve phylogeny and unambiguously places our clade of interest, Archiheterodonta as the sister group to Euheterodonta. It proves successful at resolving other problematic nodes,

especially at the base of Inaequidonta, but addition of key families, such as Thyasiridae, would be desirable to refine that part of the tree.

Where discordance of traditional taxonomic relationships of Bivalvia has persisted in the literature between hypotheses based on morphological, paleontological and molecular datasets, here we provide robust resolution of bivalve lineages, which corroborates many traditional taxonomic groups, based on disparate sources of data, from fossils to molecules, and highlights that historical discordance among bivalve classification is often not due to the choice of paleontological versus neontological; or molecular versus morphological sets of characters proper, but were contingent on basing taxonomic decisions on single character systems. Although some discrepancies persist, as for example within Protobranchia or for the derived Inaequidonta, there has been a marked stability of taxonomic groups, demonstrating concordant evolutionary signal in vastly different sources of phylogenetic data.



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## CHAPTER 3

### **A multilocus phylogeny of archiheterodont bivalves (Mollusca, Bivalvia, Archiheterodonta)**

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## Abstract

The subclass Heterodonta (Mollusca: Bivalvia) encompasses more than half of the extant bivalve species and is presently considered a derived group of the modern bivalves (Newell 1965; Waller 1998). Heterodonta is subdivided into two major groups, the hyperdiverse Euheterodonta and Archiheterodonta. The latter comprises four relatively small extant families: Astartidae, Carditidae, Condyllocardiidae and Crassatellidae, whose relationships and internal phylogeny are poorly understood. We assessed the phylogeny of archiheterodont bivalves using a multilocus dataset comprised of molecular sequence data from six loci (18S rRNA, 28S rRNA, cytochrome *c* oxidase subunit I, cytochrome *b*, internal transcribed spacer 2 and histone H3). Resultant datasets of ~4Kb of concatenated molecular sequence data were analyzed using probabilistic approaches (maximum likelihood and Bayesian inference). We recovered strong support for the monophyly of Archiheterodonta, within which Astartidae is the sister group of Crassatellidae, and these two constitute the sister clade of Carditidae, which is paraphyletic with respect to Condyllocardiidae. The relationships amongst the constituent species groups were evaluated in the context of the archiheterodont fossil record through the estimation of divergence times. Diversification times of archiheterodont families were congruent with bounded estimates of origins based on paleontological data: Archiheterodonta, 373.1 Ma (95% highest posterior density interval [HPD] 325.8-428.2); Crassatelloidea, 330.1 Ma (95% HPD 291.0-372.7); Crassatellidae, 224.0 (95% HPD 140.6-320.2); Astartidae, 288.2 Ma (95% HPD 269.2-307.3); Carditoidea, 178.8 Ma (95% HPD 120.9-228.3); Condyllocardiidae, 65.0 Ma (36.5-108.7).

## Introduction

With an estimated 8,000-20,000 species, bivalves represent the second largest living class of molluscs. The subclass Heterodonta (Mollusca: Bivalvia) encompasses the majority of extant bivalve diversity and is presently considered a derived group of the so-called “modern bivalves” (Bieler *et al.* 2013). According to Huber (2010), Heterodonta contains ca. 5,600 species out of the total 9,200 recent species of bivalves. Heterodonta is subdivided into two major groups, the hyperdiverse Euheterodonta and putatively related Archiheterodonta (Giribet 2008).

Phylogenetic evidence for a sister group relationship between Archiheterodonta and Euheterodonta is prevalent (Campbell 2000; Park & Ó Foighil 2000; Giribet & Wheeler 2002; Dryer *et al.* 2003; Giribet & Distel 2003; Taylor & Glover 2006; Harper *et al.* 2006; Taylor *et al.* 2007); notwithstanding, recent reconstructions of relationships in Bivalvia have yet to converge on the exact phylogenetic placement of Archiheterodonta (Wilson *et al.* 2010; Plazzi & Passamonti 2010; Carter *et al.* 2011; Plazzi *et al.* 2011; Sharma *et al.* 2012).

Insofar, the monophyly of Archiheterodonta is supported by molecular sequence information (Giribet & Distel 2003; Taylor *et al.* 2007; Wilson *et al.* 2010; Plazzi *et al.* 2011; Sharma *et al.* 2012), in addition to several morphological and biochemical characters, including unique sperm ultra-structure (Healy 1995) and presence of high-weight hemoglobin (Terwilliger & Terwilliger 1985); the internal phylogenetic relationships within this clade are however poorly understood (Giribet 2008). Internal phylogenies have been established for all six major lineages of similar taxonomic rank within Bivalvia; many on the basis of molecular sequence data: Protobranchia (Sharma *et al.* 2013); Pteriomorphia (e.g., Tëmkin *et al.* 2010), Paleoheterodonta (e.g., Graf & Cummings 2006), Anomalodesmata (e.g., Harper *et al.* 2006) and Heterodonta



(including Anomalodesmata) (e.g., Taylor *et al.* 2007, 2009). Owing to the contentious placement of Archiheterodonta within Bivalvia as well as the lack of internal phylogenetic resolution, Archiheterodonta remains an enigmatic clade of phylogenetic interest.

Comprising four families, Astartidae, Carditidae, Condylardiidae and Crassatellidae, Archiheterodonta species are exclusively marine, predominantly infaunal (though some byssate forms occur) suspension feeders that lack siphons. Astartidae includes four extant genera with approximately 40 species that inhabit Arctic to temperate waters (Huber 2010). Crassatellidae includes 13 living genera containing approximately 85 species and has a global distribution, though most crassatellids are found in tropical and subtropical regions (Huber 2010). Carditidae includes 16 living genera (ca. 140 species) distributed globally with the exception of the polar regions (Huber 2010). Condylardiidae, the largest family, includes 21 extant genera and about 150 species distributed globally, though little is known about the biology of its members, as most specimens are known only from dead shells (Middelfart 2002). Though no condylardiid species has been yet included in a molecular phylogenetic analysis, morphologically it has been placed within this clade (e.g., Middelfart 2002).

Among Archiheterodonta, Astartidae is usually placed with Crassatellidae in the superfamily Crassatelloidea (Chavan in Cox *et al.* 1969) on the basis of shell microstructure (Taylor *et al.* 1973) and hinge structure (Slack-Smith 1998), although the elevation of Astartidae to superfamilial status based on differences in ligament structure has been previously proposed (Bernard 1983; Coan *et al.* 2000; Huber 2010). The remaining families have been placed in Carditoidea (*sensu* Férussac 1822; Carditacea *sensu* Dall 1902), which unites Carditidae with Condylardiidae on the basis of distinguishing characteristics that include strong radial sculpture and crenulated valve margins (Slack-Smith 1998). Evaluated solely on the basis of

morphological characters, the putatively related Condyllocardiidae was revised to include two clades, Condyllocardiinae and Cuninae, each of which once was regarded as having familial rank (Middelfart 2002).

Here we investigate relationships within Archiheterodonta using molecular information from six loci (28S rRNA, 18S rRNA, internal transcribed spacer 2, histone H3, cytochrome *c* oxidase subunit I, and cytochrome *b*) to assess the phylogenetic placement and validity of the constituent families. All four families were sampled, including the first molecular sequence information for members of the family Condyllocardiidae.

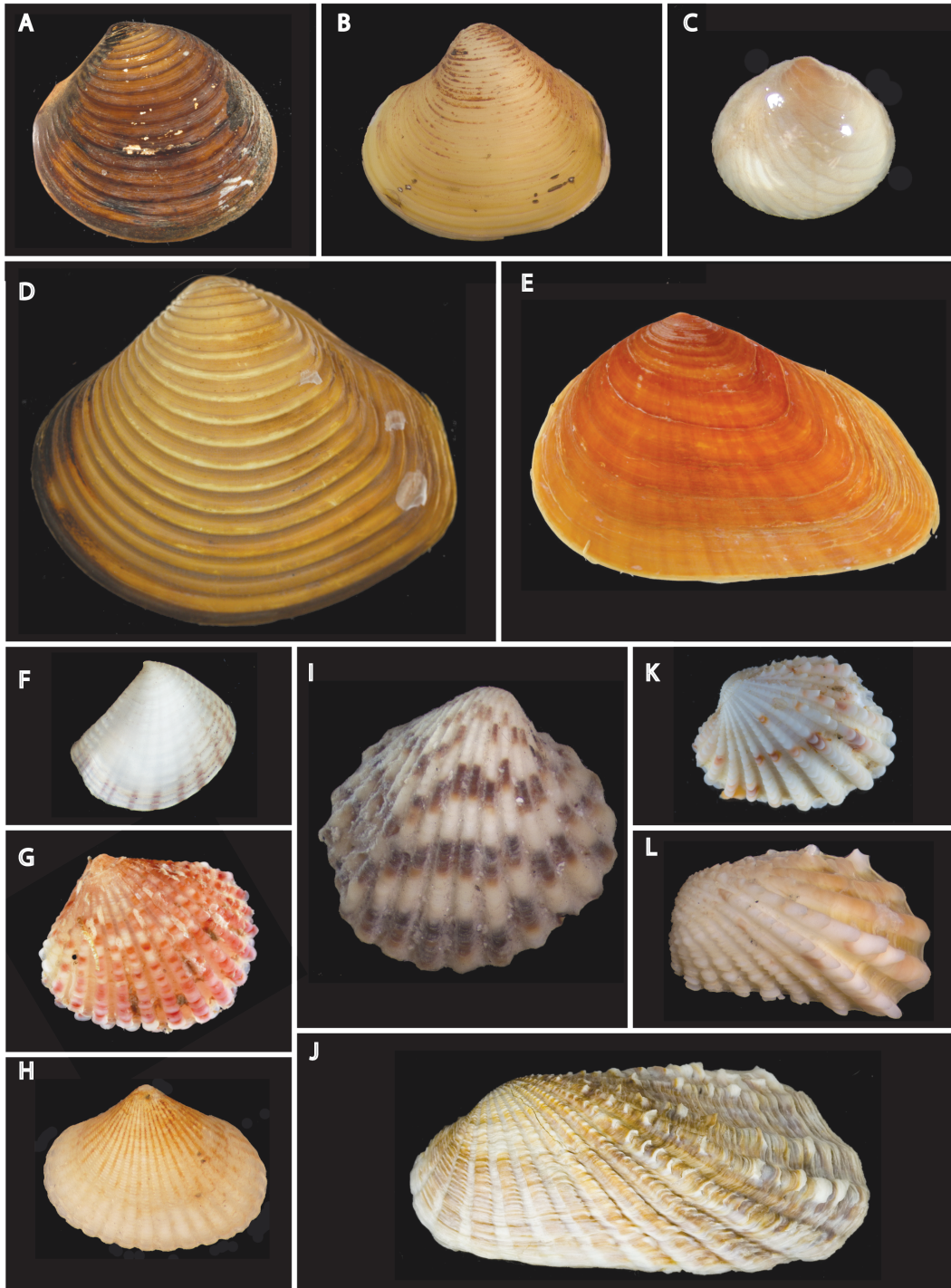
## **Materials and Methods**

### *Taxon Sampling*

Collected specimens were preserved in 96% EtOH and stored at  $-80^{\circ}\text{C}$ . The list of specimens, including voucher numbers, GenBank accession codes and collection details, is found in Table 3.1. The 45 ingroup taxa sampled consisted of eight Crassatellidae, 15 Astartidae, 20 Carditidae and four Condyllocardiidae. Exemplars of archiheterodont specimens used in subsequent analyses are shown in Figure 3.1. Outgroup taxa for the study consisted of six Palaeoheterodonta and four Euheterodonta, to represent the remaining diversity of Heteroconchia.

### *Molecular Methods*

Total DNA was extracted from either mantle or muscle tissue using Qiagen's DNEasy tissue kit (Valencia, CA, USA). Purified genomic DNA was used as a template for PCR amplification. Molecular markers consisted of two nuclear ribosomal genes and one of the ribosomal spacers (18S rRNA, 28S rRNA and internal transcribed spacer 2 [ITS2]), one nuclear protein-encoding



**Figure 3.1.** Exemplars of Archiheterodonta used in this study. Astartidae: (a) *Astarte castanea* (b) *Astarte montagui* (c) *Digitaria digitaria*; Crassatellidae: (d) *Eucrassatella cumingi* (E) *Crenocrassatella* cf. *sowerbyi* (F) *Crassinella lunulata*; Condylardiidae (G) *Carditopsis rugosa* (H) *Carditella capensis*; Carditidae: (I) *Cardites antiquata* (J) *Carditamera affinis* (K) *Megacardita pressi* (L) *Cardita distorta*.

**Table 3.1.** List of species of extant archiheterodonts sampled and gene fragments included in the phylogenetic analyses.

Taxonomy	Voucher #	28S rRNA	18S rRNA	H3	COI	ITS 2	CytB
<b>ARCHIHETERODONTA</b>							
<b>Family ASTARTIDAE d'Orbigny, 1844</b>							
<i>Goodallia triangularis</i> (Montagu, 1803)	MCZ DNA106580	XXX	X_	X		X	X
<i>Digitaria digitaria</i> (Linnaeus, 1758)	BivAToL 120.1a	XXX	XXX	X	X		
<i>Astarte undata</i> Gould, 1841	MCZ DNA105673	XXX	XXX	X	X	X	X
<i>Astarte undata</i> Gould, 1841	MCZ DNA105672	XXX	XXX	X	X	X	X
<i>Astarte sulcata</i> (da Costa, 1778)	BivAToL 192.1a	XXX	XXX	X	X		X
<i>Astarte montagui</i> (Dillwyn, 1817)	MCZ DNA106184	XXX	XXX	X			X
<i>Astarte montagui</i> (Dillwyn, 1817)	MCZ DNA106187	XXX	XXX	X			X
<i>Astarte fusca</i> (Poli, 1791)	MCZ DNA106116	XXX	XXX	X	X	X	X
<i>Astarte fusca</i> (Poli, 1791)	MCZ DNA106121	XXX		X	X		
<i>Astarte castanea</i> (Say, 1822)	MCZ DNA100142	XXX	XXX	X	X		
<i>Astarte castanea</i> (Say, 1822)	MCZ DNA105675	XXX	XXX	X	X	X	X
<i>Astarte borealis</i> (Schumacher, 1817)	MCZ DNA106182	XXX	XXX	X		X	X
<b>Family CARDITIDAE Ferrusac, 1822</b>							
<i>Cardita caliculiformis</i> Deshayes in Maillard, 1863	MCZ DNA106933	XXX	X_X	X	X	X	X
<i>Cardita caliculiformis</i> Deshayes in Maillard, 1863	MCZ DNA106934	XXX	X_X	X	X	X	
<i>Cardita caliculiformis</i> Deshayes in Maillard, 1863	MCZ DNA106935	_XX	X_	X	X	X	
<i>Cardita caliculiformis</i> Deshayes in Maillard, 1863	MCZ DNA106932	XXX	X_	X			
<i>Cardita caliculata</i> (Linnaeus, 1758)	MCZ DNA100140	XXX	XXX	X	X	X	
<i>Cardita caliculata</i> (Linnaeus, 1758)	BivAToL 119.1a	_XX	_XX	X			
<i>Cardita distorta</i> L.A. Reeve, 1843	MCZ DNA106181	XXX	XXX	X			X
<i>Cardita leana</i> W.R. Dunker, 1860	MCZ DNA101560	_XX	XXX	X	X	X	
<i>Cardita leana</i> W.R. Dunker, 1860	MCZ DNA101861	XXX	XXX	X	X	X	
<i>Cardita thaunumi</i> (Dall, Bartsch & Rehder, 1938)	MCZ DNA105741	_XX	XXX	X	X	X	
<i>Carditamera affinis</i> (G.B. Sowerby I, 1833)	MCZ DNA105641	_X	XXX	X	X	X	X
<i>Carditamera affinis</i> (G.B. Sowerby I, 1833)	MCZ DNA106149	_XX	XXX	X			
<i>Carditamera bajaensis</i> González & Giribet, 2012	MCZ DNA103800(1)	XXX	XXX	X	X	JX230972	JX230962
<i>Carditamera floridana</i> Conrad, 1838	BivAToL 31.1a	_XX	XXX	X			
<i>Cardites antiquata</i> (Linnaeus, 1758)	MCZ DNA100141	XXX	XXX	X	X		X
<i>Cardites antiquata</i> (Linnaeus, 1758)	MCZ DNA106123	_XX	XXX	X	X		X
<i>Cardites antiquata</i> (Linnaeus, 1758)	MCZ DNA106183	_XX	XXX	X	X	X	
<i>Centrocardita aculeata</i> (Poli, 1795)	MCZ DNA106112	_XX	XXX	X	X	X	
<i>Megacardita nodulosa</i> (Lamarck, 1819)	MCZ DNA103748	XXX	XXX	X	X		X
<i>Megacardita preissii</i> (K.T. Menke, 1843)	MCZ DNA106797	_XX	XXX	X	X	X	X
<i>Thecalia concamerata</i> (Bruguière, 1792)	MCZ DNA106925	XXX	X_X	X	X	X	X
<i>Thecalia concamerata</i> (Bruguière, 1792)	MCZ DNA106930	XXX	XXX	X	X	X	
<b>Family CONDYLOCARDIIDAE F. Bernard, 1892</b>							
<i>Carditella capensis</i> E.A. Smith, 1885	MCZ DNA106926	XXX	XXX	X	X	X	
<i>Carditella</i> cf. <i>similis</i> Thiele & Jaekel, 1931	MCZ DNA106928	_X	XXX	X	X		X
<i>Carditella</i> sp.	MCZ DNA pending	XXX	XXX	X	X		
<i>Carditopsis rugosa</i> (Sowerby III, 1892)	MCZ DNA106927	_XX	XXX	X	X		
<b>Family CRASSATELLIDAE Ferrusac, 1822</b>							
<i>Eucrassatella donacina</i> (Lamarck, 1818)	MCZ DNA106294	AJ581906_XX	AJ581873	X		X	
<i>Eucrassatella cumingi</i> (Adams, 1854)	BivAToL 83.2a	XXX	XXX	X	X	X	X
<i>Eucrassatella cumingi</i> (Adams, 1854)	MCZ DNA103745	XXX	XXX	X	X	X	
<i>Crenocrassatella sowerbyi</i> (Lamy, 1917)	MCZ DNA106924	XXX	XX_			X	X
<i>Crassinella lunulata</i> (Conrad, 1834)	MCZ DNA106295	XXX	XXX	X	X	X	X
<i>Crassinella lunulata</i> (Conrad, 1834)	MCZ DNA106826	XXX	XXX	X	X		X
<i>Crassatella subquadrata</i> G.B. Sowerby II, 1870	MCZ DNA106940	XXX	XXX	X	X		
<i>Crassatella crebrilirata</i> G.B. Sowerby II, 1870	MCZ DNA106923	XXX	XXX	X	X	X	X
<i>Crassatella capensis</i> Lamy, 1917	MCZ DNA106922	XXX	XXX	X	X	X	X
<i>Crassatella capensis</i> Lamy, 1917	MCZ DNA106921	XXX	XXX	X		X	X
<b>OUTGROUPS</b>							
<b>PALAEOHETERODONTA</b>							
<i>Neotrigonia margaritacea</i> (Lamarck, 1804)	AToL 100031	XXX	XXX	X	X		
<i>Neotrigonia lamarckii</i> (Gray, 1838)	BivAToL 97.1a	XXX	XXX	X	X		
<i>Aspatharia pfeifferiana</i> (Bernardi, 1860)	BivAToL 330.5a	XXX	XXX	X	X		
<i>Vesunio ambiguus</i> (Philippi, 1847)	BivAToL 172.1a	XXX	XXX	X	X		X
<i>Unio pictorum</i> (Linnaeus, 1758)	BivAToL 204.1b	XXX	XXX	X	X		X
<i>Margaritifera margaritifera</i> (Linnaeus, 1758)	BivAToL 299.2c	XXX	XXX	X	X		
<b>HETERODONTA</b>							
<b>EUHETERODONTA</b>							
<i>Mya arenaria</i> (Linnaeus, 1758)	BivAToL 18.1a	XXX	XXX	X	X		
<i>Phaxas pellucidus</i> (Pennant, 1777)	AToL	XXX	XXX	X	X		X
<i>Thracia villosiuscula</i> (MacGillivray, 1827)	AToL	XXX	XXX	X			
<i>Abra nitida</i> (O.F. Müller, 1776)	AToL	XXX	XXX	X			

gene (histone H3), and two mitochondrial protein-encoding genes (cytochrome *c* oxidase subunit I [COI] and cytochrome *b* [CYTB]). Primer sequences are listed in Table 3.2.

PCR amplifications (25  $\mu$ L) were conducted using 1  $\mu$ L of the template DNA, 1  $\mu$ L of each primer, 2.5  $\mu$ L of EconoTaq 10X PCR buffer containing 15 mM  $\text{MgCl}_2$  (Lucigen), 0.25  $\mu$ L of dNTP's 100 mM, and 1.25 U of EconoTaq DNA polymerase (Lucigen). The PCR reactions were carried out using an Eppendorf Mastercycler epgradient thermal cycler. The thermal cycle program consisted of an initial denaturation step at 95 °C for 2 min, followed by 36 cycles of denaturation at 95 °C (45 s), annealing at 43 – 53 °C (1 min) and elongation at 72 °C (90 s). The final elongation step at 72 °C (8 min) and a rapid thermal ramp for 4 °C were applied to finalize the process.

The double-stranded PCR products were visualized by agarose gel electrophoresis (1.5% agarose), cleaned with 2  $\mu$ L of diluted (1:3) ExoSAP-IT (USB Corp., Cleveland, OH, USA) in a volume of 22  $\mu$ L PCR product and performed at 37 °C for 30 min followed by enzyme inactivation at 80 °C for 15 min. Sequencing reactions were performed in a 10- $\mu$ L reaction volume using 3.2  $\mu$ L of primer (1 mM), a 1  $\mu$ L of ABI BigDye™ Terminator v. 3.0 (Applied Biosystems), 0.5  $\mu$ L BigDye 5 Sequencing Buffer (Applied Biosystems) and 3.3  $\mu$ L of cleaned PCR product. The sequencing reaction, performed by using the thermal cycler described above, involved an initial denaturation step for 3 min at 95 °C, 25 cycles (95 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min) and a rapid thermal ramp to 48 °C. The BigDye-labeled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD, USA). The chromatograms were visualized, edited and assembled using Sequencher™ (Gene Codes Corporation #1991 – 2006). External primers were cropped and discarded from the edited

sequences.

**Table 3.2.** List of primer sequences used for amplification and sequencing with original references.

Primer		Reference
<b>18S rRNA</b>		
1F	5' – TAC CTG GTT GAT CCT GCC AGT – 3'	Giribet <i>et al.</i> (1996)
4R	5' – GAA TTA CCG CGG CTG CTG G – 3'	Giribet <i>et al.</i> (1996)
3F	5' – GTT CGA TTC CGG AGA GGG – 3'	Giribet <i>et al.</i> (1996)
9R	5' – GAT CCT TCC GCA GGT TCA CCT AC – 3'	Giribet <i>et al.</i> (1996)
18Sa2.0	5' – ATG GTT GCA AAG CTG AAA – 3'	Whiting <i>et al.</i> (1997)
18Sbi	5' – GAG TCT CGT TCG TTA TCG GA – 3'	Whiting <i>et al.</i> (1997)
<b>28S rRNA</b>		
28S rd1a	5' – CCC SCG TAA YTT AGG CAT AT – 3'	Edgecombe & Giribet (2006)
28Sb	5' – TCG GAA GGA ACC AGC TAC – 3'	Whiting <i>et al.</i> (1997)
28Sa	5' – GAC CCG TCT TGA AGC ACG GA – 3'	Whiting <i>et al.</i> (1997))
28S rd5b	5' – CCA CAG CGC CAG TTC TGC TTA C – 3'	Schwendinger and Giribet (2005)
28S rd4.8a	5' – ACC TAT TCT CAA ACT TTA AAT GG – 3'	Schwendinger and Giribet (2005)
28S rd7b1	5' – GAC TTC CCT TAC CTA CAT – 3'	Schwendinger and Giribet (2005)
<b>COI</b>		
HCOout	5' – CCA GGT AAA ATT AAA ATA TAA ACT TC – 3'	Carpenter & Wheeler (1999)
LCO1490	5' – GGT CAA CAA ATC ATA AAG ATA TTG G – 3'	Folmer <i>et al.</i> (1994)
<b>Cytochrome <i>b</i></b>		
CobF	5' – GGW TAY GTW YTW CCW TGR GGW CAR AT – 3'	Passamonti (2007)
CobR	5' – GCR TAW GCR AAW ARR AAR TAY CAY TCW GG – 3'	Passamonti (2007)
<b>Histone H3</b>		
H3F	5' – TTY TGN GCR TTC CAD ATC AT – 3'	Colgan <i>et al.</i> (1998)
H3R	5' – YTK ATH AAR GCT ATG GA – 3'	Colgan <i>et al.</i> (1998)
<b>Internal Transcribed Spacer 2</b>		
5.8Sfor	5' – GAA TCA TCG AGT CTT TGA ACG C – 3'	Murienne <i>et al.</i> (2011)
28Srev	5' – GTT AGT TTC TTT TCC TCC GCT T – 3'	Murienne <i>et al.</i> (2011)

### *Phylogenetic Analysis*

Model-based (maximum likelihood, Bayesian) approaches were used to infer phylogenetic relationships. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on static alignments. Static alignments were generated with MUSCLE ver. 3.6 (Edgar 2004) with default parameters. Length invariable sequences (COI, CYTB) were confirmed using amino acid sequence translations to ensure that no treatment with MUSCLE ver. 3.6 was required. Static alignments for length variable datasets were treated with GBlocks v. 0.91b (Castresana 2000) to



eliminate hypervariable regions from the nucleotide sequence alignments. Sequence lengths prior and after treatment with Gblocks are listed in Table 3.3.

**Table 3.3.** Alignment sizes prior to and after treatment for length variability in GBlocks.

Partition	Original length of alignment (bp)	Fraction retained by Gblocks (%)	Final length of alignment (bp)
28S rRNA	2396	76	1844
18S rRNA	1881	91	1713
COI	664	94	627
H3	327	100	327
CytB	389	100	389
ITS2	728	28	225

ML analyses were conducted using RAxML ver. 7.2.7 (Stamatakis 2006) on 96 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu). For the maximum likelihood searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR +  $\Gamma$ ) was specified for each data partition, and 100 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates) using the GTR model (Stamatakis *et al.* 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches.

BI analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck & Ronquist 2005) on 96 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu), with a unique model of sequence evolution with corrections for a discrete gamma distribution and/or a proportion of invariant sites on each partition, as selected in jModeltest ver. 0.1.1 (Posada 2008; Guindon & Gascuel 2003) under the Akaike Information Criterion (Posada & Buckley 2004). Default priors were used starting with random trees, and

three runs, each with three hot and one cold Markov chains, were conducted until the average deviation of split frequencies reached  $< 0.01$  ( $10^9$  generations). Stationarity was checked using Tracer ver. 1.5 (Rambaut & Drummond 2009). After burn-in samples were discarded, a majority-rule consensus topology was generated from the sampled trees.

#### *Estimation of divergence times*

Ages of clades were inferred using BEAST ver. 1.6.1 (Drummond *et al.* 2006; Drummond & Rambaut 2007). We specified a unique GTR model of sequence evolution with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + G + I) for each partition.

Three fossil taxa were used to calibrate divergence times. The diversification of Astartidae was constrained with the early Permian fossil (Pennsylvanian stage,  $298.9 \pm 0.8$  Ma) *Astartella vera* Hall, 1958, placed originally in Crassatelloidea, comprised of Astartidae and Crassatellidae (Cox *et al.* 1969), and later revised to Astartidae on the basis of shell morphology (Nicol 1955). To account for uncertainty we applied a normal distribution prior to this node with a mean of 298.9 and a standard deviation of 10 Myr. Identifying suitable constraints for stem group members of Carditidae was problematic. Chavan (in Cox *et al.* 1969) placed *Permophorus* Chavan, 1954 as a lower Carboniferous to Permian genus of Carditoidea (Cox *et al.* 1969; Slack-Smith 1998) in the family Permophoridae van de Poel, 1959. While permophorids were once considered to be members of Carditidae (e.g., Cox *et al.* 1969; Slack-Smith 1998), current classifications place them in Anomalodesmata (Kelley *et al.* 2000). The minimum age for the split of Anomalodesmata from Inaequidonta was constrained using a normal distribution prior spanning 478.6-488.3, based on *Ucumaris conradoi* Sánchez & Vaccari, 2003 (from the Tremadocian). The minimum age for the diversification of Unionoidea

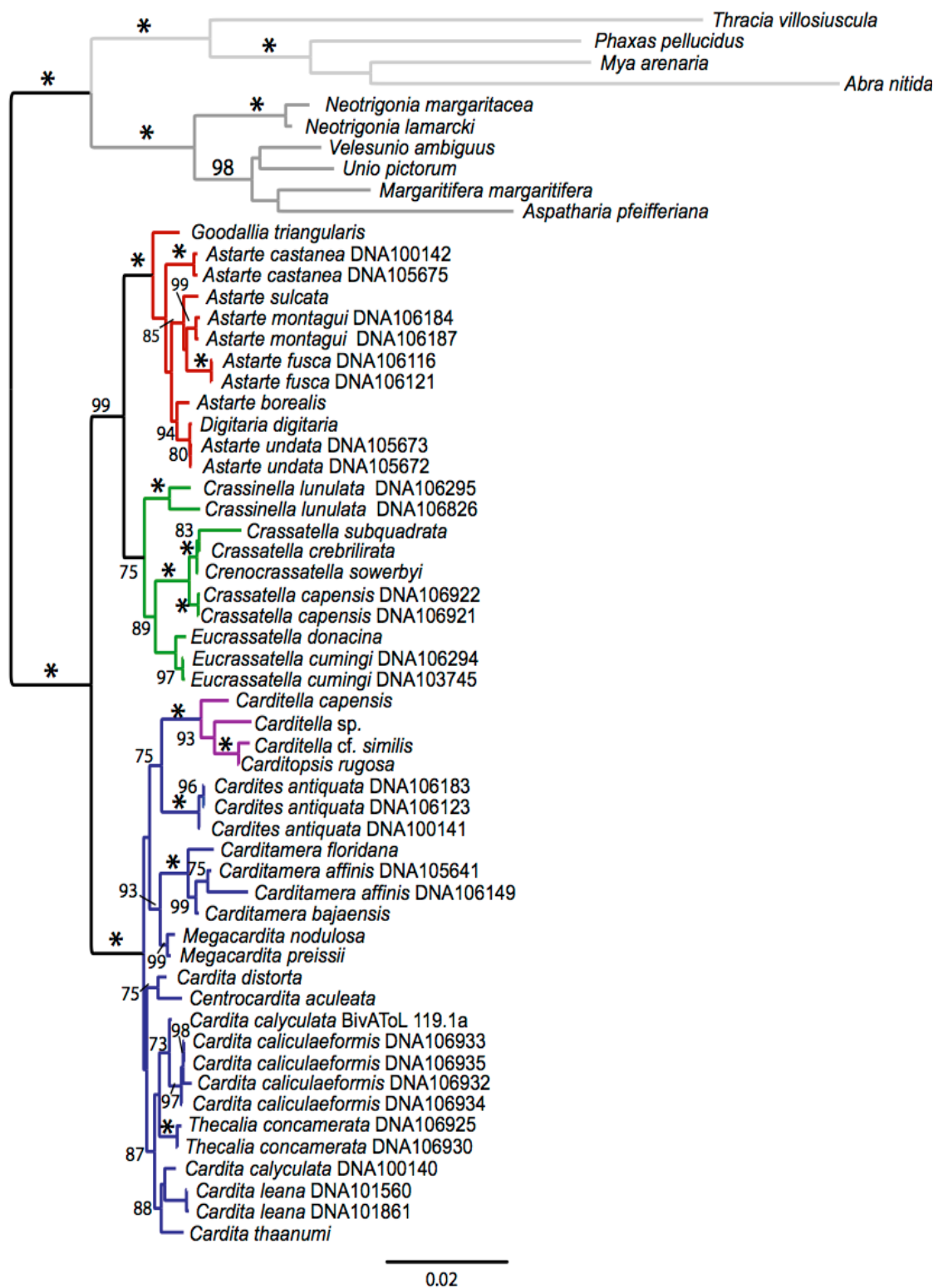


was constrained with the Late Jurassic fossil *Hadrodon jurassicus* Yen, 1952, whose date has been inferred as the base of the Tidwell Member at 154.9 Mya (Kowallis *et al.* 1998). To account for uncertainty we applied a normal distribution prior to this node with a mean of 152 and a standard deviation of 5 Myr. Divergence time calibration for the root node drew upon a previous study of the class Bivalvia, wherein molecular dating was conducted using the same methodology and constrained using fossil taxa (Bieler *et al.* *in press*). Here, we employed transitive dating and took the 95% HPD intervals to constrain the root node (95% HPD 509.5-521.7). A normal distribution prior was used to calibrate this node with a mean of 515.9 and a standard deviation of 3 Myr. An uncorrelated lognormal clock model was inferred for each partition, and a Yule speciation process was assumed for the tree prior (Drummond *et al.* 2006). Markov chains were run for 50,000,000 generations, sampling every 5000 generations. Convergence diagnostics were assessed using Tracer ver. 1.5 (Rambaut & Drummond 2009).

## Results

### *Maximum likelihood*

Maximum likelihood analysis of the six-gene, 45-ingroup taxon dataset resulted in an optimal tree topology with  $\ln L = -36729.78$  (Figure 3.2). The recovered topology showed monophyly of Archiheterodonta (BS = 100); monophyly of Crassatelloidea (BS = 99) and the mutual monophyly of its constituent sister clades, the strongly supported Astartidae (BS = 100) plus Crassatellidae (BS = 75). Monophyly of Carditoidea (Carditidae + Condyllocardiidae) was also strongly supported (BS = 100), however, monophyly of Carditidae was not recovered, as it includes the four representative condyllocardiids. Condyllocardiidae, represented by exemplars of Carditellinae (*Carditella*) and Condyllocardiinae (*Carditopsis*), was monophyletic (BS = 100).



**Figure 3.2.** Phylogenetic relationships of Archiheterodonta based on maximum likelihood analysis of six genes ( $\ln L = -38412.02$ ). Numbers on nodes indicate bootstrap resampling frequencies. Colors in tree topology correspond to the four traditional families (red: Astartidae; green: Crassatellidae; blue: Carditidae; purple: Condyllocardiidae).

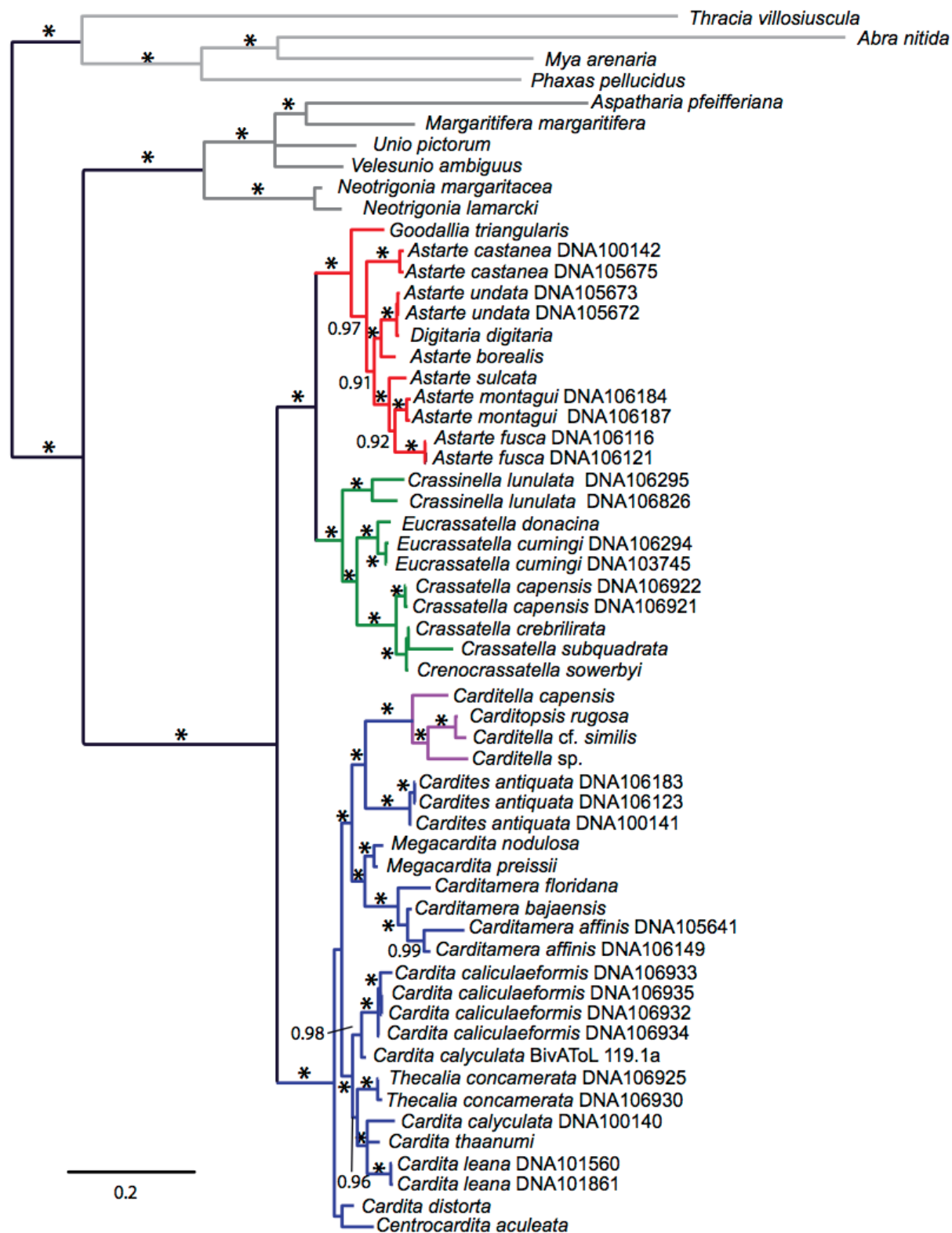
Within Astartidae, *Astarte*, the only genus represented by multiple specimens, was paraphyletic, as it includes *Digitaria*; *Goodallia* is the sister group to the paraphyletic *Astarte*. All *Astarte* species represented by multiple specimens were recovered as monophyletic.

Among Crassatellidae, genera represented by multiple specimens were monophyletic with the exception of the *Crassatella*, which included *Crenocrassatella sowerbyi*. *Crassinella* is the sister group to the remaining representatives of the family, and *Eucrassatella* is the sister genus to the *Crassatella*—*Crenocrassatella* assemblage.

Within Carditidae several genera were recovered as monophyletic (*Cardites*, *Carditamera*, *Megacardita*, *Thecalia*), but *Cardita* was polyphyletic. Likewise, *Cardita calyculata*, represented by multiple individuals, was not monophyletic, however some specimens were missing fragments of the six loci (see discussion below; Table 3.1).

#### *Bayesian inference*

The Bayesian inference recovered a topology highly congruent at the familial level with that of the ML analysis, with monophyletic Archiheterodonta (PP = 1.00), Crassatelloidea (PP = 1.00), Astartidae (PP = 1.00), Crassatellidae (PP = 1.00), Carditoidea (PP = 1.00) and Condyllocardiidae (PP = 1.00) (Figure 3.3). Topological differences with the ML analysis are prevalent within Carditidae. The *Cardita distorta* + *Centrocardita aculeata* clade differ from the maximum likelihood topology, which recovers this lineage in a derived placement sister to *Carditamera*, while it is sister to all other species in the Bayesian analysis. Generally, nodes that are highly supported in both topologies were congruent.



**Figure 3.3.** Phylogenetic relationships of Archiheterodonta based on Bayesian inference analysis of five genes. Numbers on nodes indicate posterior probabilities. Colors in tree correspond to the four traditional families (as in Fig. 3.2).

### *Estimation of divergence times*

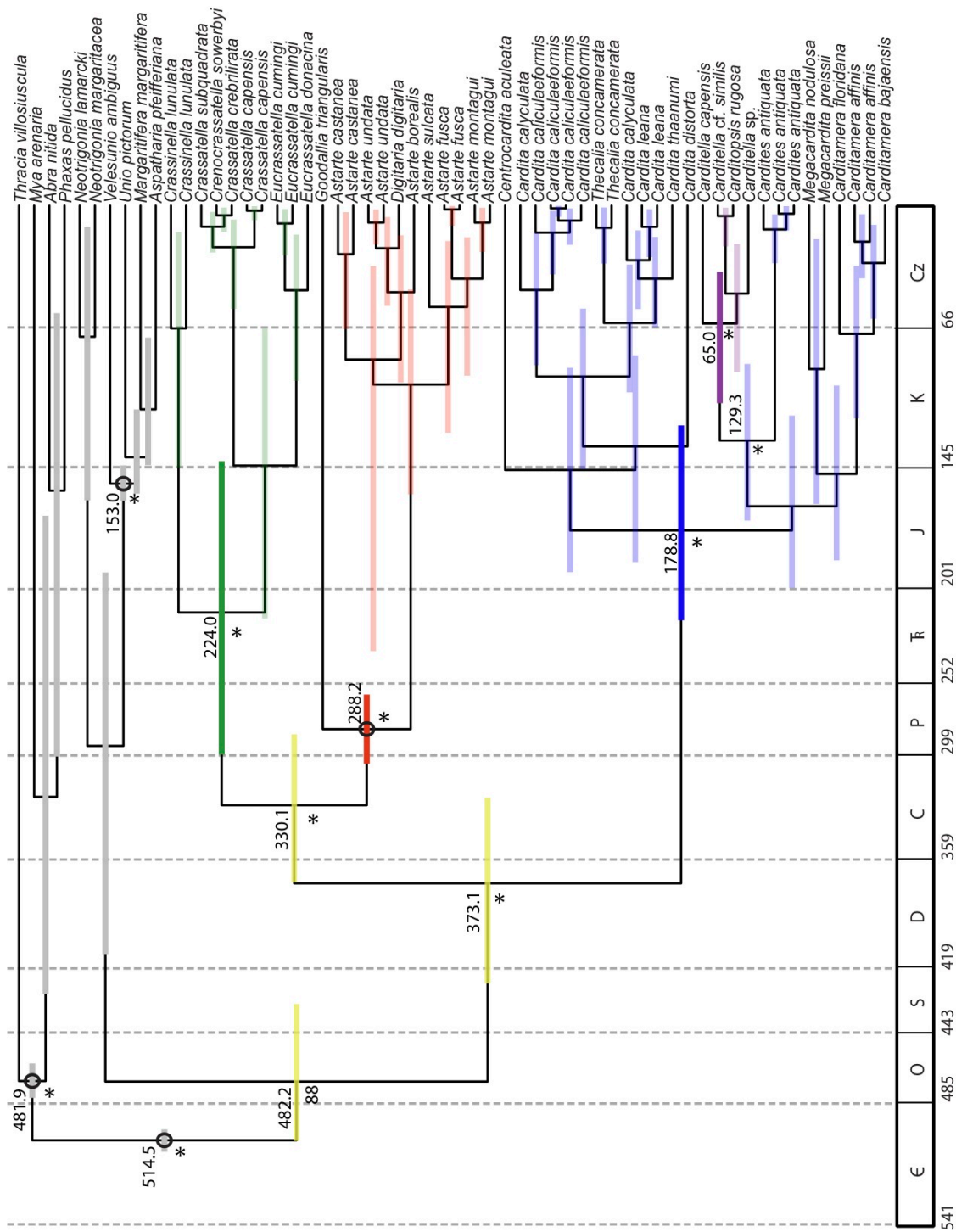
The tree topology recovered differed from congruent nodes recovered from those both previous analyses. Diversification of major lineages using BEAST was estimated as follows:

Archiheterodonta, 373.1 Ma (95% highest posterior density interval [HPD] 325.8-428.2); Crassatelloidea, 330.1 Ma (95% HPD 291.0-372.7); Crassatellidae, 224.0 (95% HPD 140.6-320.2); Astartidae, 288.2 Ma (95% HPD 269.2-307.3); Carditoidea, 178.8 Ma (95% HPD 120.9-228.3); Condyllocardiidae, 65.0 Ma (36.5-108.7) (Figure 3.4).

### **Discussion**

This study represents the first comprehensive molecular phylogenetic analysis of Archiheterodonta. Previous molecular phylogenies included representatives of no more than two species per family and from only three families, Astartidae, Carditidae and Crassatellidae. For the first time molecular sequence information was included for members of Condyllocardiidae, whose placement within Archiheterodonta is strongly supported in all analyses. However, the validity of the family is questionable, as Condyllocardiidae nests within the Carditidae (Figures 3.2-4), but no member of the nominal genus *Condyllocardia* was sampled, and until this is done, no taxonomic action will be taken.

Prior molecular analyses have highlighted a close relationship of Astartidae and Carditidae (e.g., Giribet & Wheeler 2002; Giribet & Distel 2003), Astartidae and Crassatellidae (e.g., Sharma *et al.* 2012) and Astartidae, Carditidae and Crassatellidae (e.g., Taylor *et al.* 2007). Dall (1902) also hypothesized these families were related based on paleontological history,



**Figure 3.4.** Evolutionary divergence times of Archiheterodonta inferred with BEAST for all molecular data. Colored bars indicate 95% highest posterior density intervals for nodes of interest. Black text above selected nodes indicates median ages; text below branches indicates posterior probabilities. Open circles indicate calibrated nodes.

anatomy and development. Within Archiheterodonta, interfamilial relationships recovered confirm previous taxonomic hypotheses based on morphology, that Crassatelloidea (Astartidae + Crassatellidae) and Carditoidea (Carditidae + Condyllocardiidae) are closely related (e.g., Cox 1960; Yonge 1969; Healy 1995). These results thus corroborate the suggested union of Crassatelloidea (including Astartidae) and Carditoidea proposed by Yonge (1969) and Healy (1995) and supported by Taylor *et al.* (2007), among other recent analyses including multiple heterodont groups.

Additionally, the occurrence of lineage specific hemoglobin within Archiheterodonta is a captivating synapomorphy of this group (Terwilliger & Terwilliger 1985). The presence of hemoglobin in tissues is found throughout the Mollusca and has been documented in several protobranch and heterodont species (Alyakrinskaya 2003) as well as in species of Pteriomorphia (Terwilliger & Terwilliger 1983). Hemoglobins have been found to be predominantly concentrated in the gills and the adductor muscle, though can be present in the mantle, foot, nervous tissue, heart and exhalant siphon (Alyakrinskaya 2003). Within Bivalvia, hemoglobins are usually small and intracellular (Lieb *et al.* 2006). However, hemoglobins characteristic of *Astarte* (Astartidae) and *Cardita* (Carditidae) are known to be large 14 to 24 domain rod-like hemoglobins (Terwilliger & Terwilliger 1983; Lieb *et al.* 2006), and have been observed in *Eucrassatella* (Crassatellidae) and *Crassinella* (Crassatellidae) (Taylor *et al.* 2007). Although present in all archiheterodont families assessed, further evaluation is required to establish functional significance of these archiheterodont specific hemoglobins (Taylor *et al.* 2007).

### *Crassatelloidea, Férussac 1822*

All inferences of tree topology recover a clade comprised of Astartidae and Crassatellidae (the traditional Crassatelloidea *sensu* Férussac, 1822), which is supported morphologically by several features: similar ctenidial structure, in that two non-plicate demibranchs comprised of simple filaments are present; similarity of the labial palps, ridged and triangular; and the degree of mantle fusion, where the mantle in these families is only fused at the exhalant siphon (Saleuddin 1965). Differences in ligament structure have been used to justify the elevation of Astartidae and Crassatellidae as separate superfamilies (Bernard 1983; Coan *et al.* 2000), something that seems unnecessary under the current phylogenetic results. Here, all analyses support the mutual monophyly of these families (Figures 3.2-4) and as such, results do not corroborate the elevation of taxonomic rank of these families.

### *Carditoidea, Férussac 1822*

All phylogenetic analyses unambiguously recover Carditoidea (Carditidae + Condyllocardiidae: *sensu* Férussac, 1822; Carditacea *sensu* Dall, 1902), which is supported by the distinguishing shell characteristics including strong radial sculpture, crenulated valve margins and the absence of the sinus in the pallial line (Slack-Smith 1998). Huber (2010) recognizes a third family placed within Carditoidea, Cardiniidae – an mostly fossil family with a sole extant member, *Tellidorella cristulata* Berry, 1963 - a small species resembling *Crassinella*. This species was previously postulated to be related to Crassatellidae, despite having an external ligament (Berry 1963) and was then revised to be included in Astartidae (Ollson 1964). While, Chavan (in Cox *et al.* 1969) resurrected this otherwise extinct family Cardiniidae to be represented by a small unique Panamic species, a so called "living fossil." Since then, it has been discovered to actually be



more closely related to the Lucinidae where it was recently transferred (Taylor *et al.* 2011; see also Coan & Valentich-Scott 2012). Though Cardiniidae is identified as a valid extant family of Carditidae (Huber 2010; Bieler *et al.* 2010), we follow the more recent work of Taylor *et al.* (2007) and do not consider it as a member of Archiheterodonta.

Molecular sequence information for members of Condyllocardiidae has never been included in a published work, and here we recover Condyllocardiidae nested within Carditidae. Condyllocardiidae are distinguished morphologically by the absence of an outer demibranch, solid ovate shells and umbones with a large prominent prodissoconch (Middelfart 2002; Huber 2010), but anatomical data are missing for most named species. The placement of two condyllocardiid genera, *Carditella* and *Carditopsis*, within Carditidae was recovered regardless of algorithmic treatment. Güller & Zelaya (2013) found both genera *Carditella* and *Carditopsis*, here studied, as well as *Cyclocardia* species (Carditidae), to have both inner and outer demibranchs. The absence of an outer demibranch, a synapomorphy of Condyllocardiidae used to distinguish these super-families, may actually be correlated to small size, and not restricted to Condyllocardiidae (Güller & Zelaya 2013). Though no taxonomic action is proposed for the family Condyllocardiidae, results from the analyses here presented support members of *Carditella* and *Carditopsis* to be nested within Carditidae. Condyllocardiidae is currently divided up into three subfamilies, Carditellinae Kuroda, Habe & Oyama, 1971, Condyllocardiinae Bernard, 1886 and Cuninae Lamprell & Healy, 1998, and we include representatives of Carditellinae (*Carditella*) and Condyllocardiinae (*Carditopsis*), finding *Carditopsis* nested within *Carditella*, rendering the latter genus and the subfamily Carditellinae paraphyletic. Although further evaluation including the type genus of the family is needed before taking taxonomic

action, our data strongly suggest that Condyllocardiidae is not a valid family, as it is deeply nested within Carditidae.

### *Intrafamilial relationships*

Intrafamilial congeneric relationships were evaluated for each of the four families, however no taxonomic action has been proposed here due to limited sampling within each clade.

Forthcoming systematic revisions within Archiheterodonta are imperative for establishing new generic classifications based on natural, monophyletic groups. For genera represented by multiple specimens, relationships recovered in our analyses are consistent with traditional hypotheses based on morphological studies, but there are some unexpected results, which are discussed here.

Astartidae are distinguished by their solid, thick triangular to elliptical shells, weak to strong commarginal sculpture with prominent co-marginal ridges, as in *Astarte* (Figures 3.1a,b), or of oblique sculpture as in *Digitaria* (Figure 3.1c), thick periostracum and hinge dentition with two or three prominent cardinals in each valve; laterals weak or absent (Huber 2010). The genus *Astarte* was paraphyletic in all analyses as it includes *Digitaria*; *Astarte* comprises 32 species, being the largest genus in the family, while the genera *Digitaria* and *Gonilia* include two species each, and *Goodallia* includes five (see Giribet & Peñas 1999). *Goodallia*, represented in our analyses by a single species, constitutes the sister group of the *Astarte-Digitaria* clade. Difficulty in identifying species of *Astarte* has been attributed to ambiguous morphological characters used to delimit taxa within this genus (Petersen 2001). However, here we recover all *Astarte* species represented by multiple individuals monophyletic, with *Digitaria* closely related to *A. undata*.

Within Crassatellidae, each species represented by multiple individuals was found monophyletic. *Eucrassatella* was also monophyletic, but *Crassatella* was paraphyletic by virtue of *Crenocrassatella sowerbyi* nesting within this clade. It is unclear if our phylogenetic relationships reflect the distribution of morphological apomorphies as this group is largely understudied and little is known of the anatomy and biology of most Crassatellidae, with the exception of *Crassinella lunulata* (Taylor *et al.* 2007).

Within Carditidae, the monophyly of *Carditamera* and *Megacardita* were recovered with strong support. These genera along with *Cardita* were represented by multiple species, but *Cardita* was polyphyletic. Species of *Cardita* are distinguished by the degree of presence or absence of hinge teeth and external shell morphology, where the *Cardita* hinge has two left and three right cardinals and obsolete laterals; which differs from *Carditamera* in the laterals are well developed and the right anterior cardinal is obsolete (Dall 1902). Though species-level delimitations are well established, assigning species to genera within this family is difficult as morphological definitions of these genera overlap (Coan & Valentich-Scott 2012).

Condylocardiidae are small in size, whose genera are distinguished by hinge teeth, external ornamentation and the possession of an internal ligament. Within Condylocardiidae, *Carditella* was paraphyletic with respect to *Carditopsis rugosa*, nesting within this clade. Recent redescriptions of the type species of *Carditella* and *Carditopsis* highlight the need for a revision of species attributed to these genera, as consistency of distinguishing morphological characteristics was not observed (Güller & Zelaya 2013). Specifically, differences in the extension and location of the resilifer were noted for *Carditella* and differing hinge morphology of species described in *Carditopsis* by multiple authors (Güller & Zelaya 2013).

### *Divergence Times*

The origin of Archiheterodonta is postulated to be as early as the Ordovician or at least in the Devonian, where diversification of each of these major clades occurred during the Permian (Cox *et al.* 1969). Dates recovered place the origin of Archiheterodonta in the Ordovician at 482.2  $\pm$  75.4 Ma (BS = 88). A gap of 160 Ma between the origin and diversification of the clade is present, suggesting that the diversification of this group was not constant throughout evolutionary time. The split between Crassatelloidea and Carditoidea is in the Devonian at 373.1 Ma (BS = 100). The diversification of extant lineages pre-date those suggested through paleontological records of each of the four families, which could be attributed to sampling artifacts, as the diversity of all extant lineages was not included in these analyses. Although Crassatellidae are not very diverse at the present day, they have been postulated to be a prominent component of benthic communities in the Cretaceous (Taylor *et al.* 2007), which is concordant with the results presented here. Diversification of Condyllocardiidae supports suggested origins in the Eocene (Cox *et al.* 1969).

### **Conclusions**

The present study robustly recovers the monophyly of Archiheterodonta, including molecular sequence information from all four families for the first time. Of principal interest are the phylogenetic placement of Condyllocardiidae, clearly nested within Carditidae, and the systematic validity of the included superfamilies. Many of the relationships recovered in our analyses are consistent with traditional hypotheses, as all phylogenetic analyses based on molecular sequence data unambiguously recover with significant support the division of Archiheterodonta into two clades, corresponding to Crassatelloidea (Astartidae + Crassatellidae)

and Carditoidea (Carditidae + Condyllocardiidae) (Figures 3.2–4). Finally, estimated divergence times are concordant with established diversification times based on the fossil record of Archiheterodonta.

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## CHAPTER 4

### **A new cryptic species of carditid bivalve from the Gulf of California (Mollusca, Bivalvia, Archiheterodonta, Carditidae)**

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## Abstract

*Carditamera bajaensis*, new species, is described from semi-infaunal specimens collected in the intertidal zone in the Golfo de California, Baja California Sur, Mexico. The new species resembles *Carditamera affinis* (G. B. Sowerby I, 1833), the only valid *Carditamera* species known from within the Golfo de California, with which it has been mistaken, but it differs in shell structure and most conspicuously in life mode – semi-infaunal for *C. bajaensis* versus byssally attached to hard substrata for *C. affinis*. Haplotype networks constructed from two mitochondrial genes (16S rRNA and cytochrome b) and one nuclear gene (internal transcribed spacer 2) indicate a clear genetic break between *C. affinis* and *C. bajaensis*, as suspected initially due to their different modes of life and shell morphology. This pair of species, *C. affinis* and *C. bajaensis*, overlapping in distribution yet genetically distinct, possibly indicate ecological speciation.

Key words: Cryptic species, Carditidae, Carditamera, Baja California, Archiheterodonta, haplotype networks.

## Introduction

Western North America has a particularly species-rich marine bivalve fauna (Coan et al., 2000; Coan & Valentich-Scott, 2012), especially with regard to members of Carditidae. Carditid bivalves are exclusively marine, predominantly infaunal (though some byssate forms occur), suspension-feeders that lack siphons. Carditids are one of the neglected larger families of bivalves; the last genus-level systematic treatment of the Carditidae was given by Chavan (1969), and only 50 species were ascribed to this group by Boss (1982). Recently, 92 species were illustrated by Huber (2010), who recognized approximately 140 species in the family.

Carditidae are postulated to have originated in the Ordovician and have been recorded as early as the Devonian (Chavan, 1969), though the lineage diversified much later in the Cretaceous (Slack-Smith, 1998). The development and reproductive biology of Carditidae was examined by Dall (1902). Most species are brooders, in which juveniles are retained within the body cavity of the female until the process of secretion of the calcified shell has commenced, along with the complete formation of the prodissoconch (Mikkelsen & Bieler, 2008). Though carditids predominantly inhabit shallow water, several members, such as the mainly Arctic and boreal *Cyclocardia*, have been collected at depths of up to 1,707 m (Dall, 1902).

Phylogenetically, Carditidae clusters with three other basal heterodont bivalve families – Astartidae, Crassatellidae, and Condyllocardiidae – constituting the well-supported clade Archiheterodonta (Taylor et al., 2007; Giribet, 2008). Though no condyllocardiid species has been yet included in a molecular phylogenetic analysis, morphologically it has been placed within this clade (Middlefart 2002). The Astartidae, a smaller family, includes four living genera with approximately 40 species that inhabit Arctic to temperate waters (Huber, 2010). The Crassatellidae, a moderately sized family,

includes 13 living genera containing approximately 85 species and has a global distribution, though most crassatellids are found in tropical and subtropical regions (Huber, 2010). Condyllocardiidae, a larger family, includes 21 living genera and about 150 species distributed globally, though little is known about the biology of its members (Middlefart, 2002). Phylogenetic evidence for a sister relationship between Archiheterodonta and the remainder of Heterodonta (Euheterodonta) is commonly recovered (Campbell, 2000; Park & Ó Foighil, 2000; Giribet & Wheeler, 2002; Dryer et al., 2003; Giribet & Distel, 2003; Taylor & Glover, 2006; Harper et al., 2006; Taylor et al., 2007, 2009), but a sister group to Palaeoheterodonta has also been suggested (Sharma et al., 2012). The relationships within Archiheterodonta remain however unresolved (Giribet, 2008) but its monophyly is further supported by several morphological character systems, such as sperm ultrastructure (Healy, 1995). Hemoglobin has also been reported in several members of this clade (e.g., Taylor et al., 2005; Terwilliger & Terwilliger, 1985). Within Carditidae, extracellular hemoglobin has been reported in the blood of *Carditamera affinis* (G. B. Sowerby I, 1833).

The genus *Carditamera* Conrad, 1838, originally described from fossil specimens, can be distinguished from other members of Carditidae by an equivalve, oblong shell as well as hinge morphology, in which both strong cardinals and well-developed laterals are present, and is known to the Eocene (Conrad, 1838). As part of an ongoing systematic revision of Carditidae (VLG, work in progress) analysis of the phylogenetic breadth of this group was undertaken. Specifically, in examining species delimitations within Carditidae, molecular information elucidated species-level divergences within *Carditamera*, and thus, we report data that support the discovery of a new species.

*Carditamera affinis* was originally described from the Golfo de Nicoya, Costa Rica, but its distribution is said to extend north through Baja California and as far south as Peru (Huber, 2010). The morphological variability ascribed to *Carditamera affinis* is uncharacteristic of other species in the genus and was therefore reexamined for the possibility of additional species. Here, a new species of Carditidae belonging to the genus *Carditamera* is described from specimens previously considered *Carditamera affinis* (Figs. 4.1–4.3).

## Materials and Methods

### *Abbreviations*

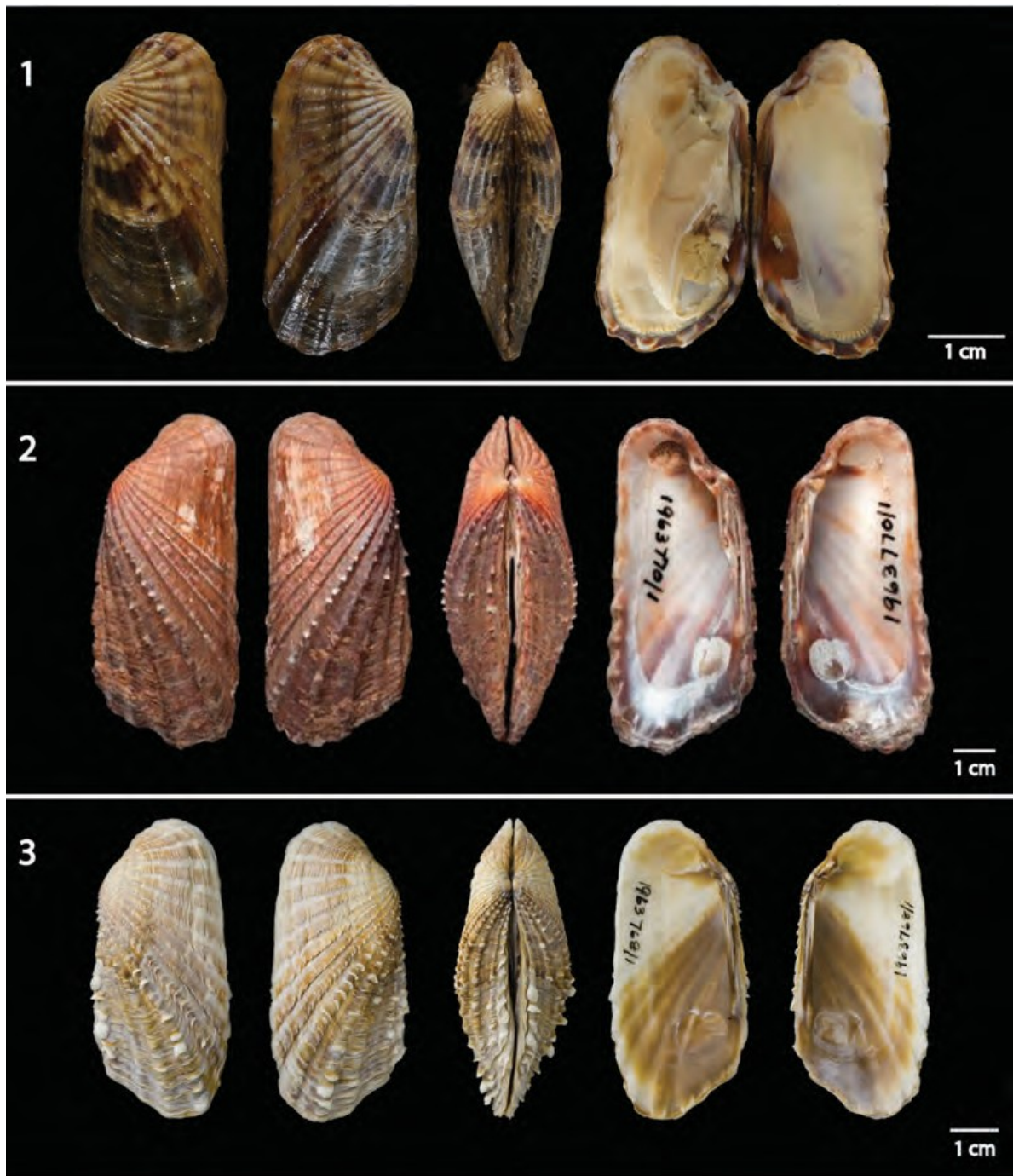
Specimens are housed in the following institutions:

MCZ            Museum of Comparative Zoology, Harvard University, Cambridge,  
Massachusetts, U.S.A.

NHMUK        The Natural History Museum, London, England, U.K.

### *Identification*

*Carditamera* includes six species, but only *C. affinis* (Fig. 4.2) and *C. radiata* (G. B. Sowerby I, 1833) (Fig. 4.4) are known from tropical western Mexico. *Carditamera affinis* overlaps in distribution range with the new species reported here. *Carditamera affinis* can be distinguished by very thin-to-broad radial ribs, with scales or small spines on the posterior ends of the larger ribs. *Carditamera radiata* is not known from the Golfo de California, however it does overlap with described distribution of *C. affinis* (Coan & Valentich-Scott, 2012). *Carditamera radiata* has smooth broad ribs, a short anterior end, a strongly tapering posterior end and has a maximal size



**Figure 4.1–4.3.** Shells of *Carditamera* spp. FIG. 4.1: *Carditamera bajaensis*, sp. nov. (MCZ DNA106146\_1) collected in La Paz, Baja California Sur, Mexico; FIG. 4.2: *Carditamera affinis* (G. B. Sowerby I, 1833) s syntype (NHMUK) from the Golfo de Nicoya; FIG. 4.3: *Cardita californica* Deshayes, 1854, syntype (NHMUK) from the Golfo de California.



of 56 mm. Huber (2010: 252) illustrated the new species under the name *Carditamera affinis*; Coan & Valentich-Scott (2012) used a illustrations of the new species for both *C. affinis* and *C. radiata*.



**Figure. 4.4** *Carditamera radiata* (G. B. Sowerby I, 1833), syntype (NHMUK) collected in Salango, western Colombia and Panama from muddy sand from 11 to 18 m depth.

### *Anatomy*

All specimens were preserved in ~96% ethanol for molecular work, as they were originally were thought to be *Carditamera affinis*. No specimens suitable for histological study are thus available (Table 4.1).

### *Molecular Analysis*

A total of 11 specimens were used in the molecular analysis (Table 4.1): nine *Carditamera* specimens collected in February 2009 from Bahía Balandra (24°19.019'N, 110°19.27'W) near

La Paz, Baja California Sur (six from a sandy bottom substratum and three byssally attached to rocks); and two specimens collected in March 2003 from Bahía la Choya (31°20'37''N, 113°38'38''W), Puerto Peñasco, Sonora, Golfo de California, found in the intertidal zone, buried in sand. Specimens and subsequent DNA extractions were retained as vouchers and are deposited in the Museum of Comparative Zoology, Department of Invertebrate Zoology DNA collection.

**Table 4.1.** List of MCZ accession numbers, collecting localities, and GenBank accession numbers for specimens used in molecular analysis.

MCZ Accession	Collection Site	ITS 2	16S rRNA	CYT B
103800_1	Golfo de California, Puerto Peñasco, Bahía la Choya, Sonora, Mexico, G. Giribet, T. Hardy, M. K. Nishigushi	**	**	JX230961
103800_2	"	JX230972	JX230955	JX230962
106146_1	Golfo de California, Bahía Balandra, La Paz, Baja California Sur, Mexico, V. González & G. Kawauchi, 28.XII.2009 [24°19.019'N, 10°19.27'W]	***	***	JX230963
106146_2	"	***	***	JX230964
106146_3	"	***	***	JX230965
106146_4	"	JX230973	JX230956	JX230966
106146_6	"	JX230974	JX230957	JX230967
106146_7	"	JX230975	JX230958	JX230968
106147_1	"	JX230976	JX230959	JX230969
106147_2	"	JX230977	JX230960	JX230970
106147_3	"	***	***	JX230971

Total DNA was extracted from a small tissue sample from the mantle or foot using the DNeasy Tissue Kit (Qiagen) and the protocol provided by the manufacturer. The purified DNA was used as a template for PCR amplification of fragments of two mitochondrial genes (16S rRNA, and cytochrome b) and one nuclear gene (internal transcribed spacer region 2; ITS-2). The 16S rRNA gene was amplified and

**Table 4.2.** List of specimens examined for morphological comparisons, including MCZ accession numbers, collecting localities, species, and number of specimens.

MCZ Accession No.	Collection Site	Species	No. of Specimens
DNA 103800	Golfo de California, Puerto Peñasco, Bahía la Choya, Sonora, Mexico, G. Giribet, T. Hardy, M. K. Nishigushi 27.III.2003 [31°20'37"N, 113°38'38"W]	<i>C. bajaensis</i>	2
DNA 106146	Golfo de California, Bahía Balandra, La Paz, Baja California Sur, Mexico, V. González & G. Kawauchi, 28.XII.2009 [24°19.019'N, 110°19.27'W]	<i>C. bajaensis</i>	7
DNA 106147	Golfo de California, Bahía Balandra, La Paz, Baja California Sur, Mexico, V. González & G. Kawauchi, 28.XII.2009 [24°19.019'N, 110°19.27'W]	<i>C. affinis</i>	3
96	Golfo de California, N. W. Lermond Collection Acc. 665, C. R. Orcutt, collection date unknown	<i>C. affinis</i>	1
1300	Mazatlán, Mexico, W. J. Eyerdam, 14.XIII.1938	<i>C. affinis</i>	3
21674	Panama, E. R. Mayo, collection date unknown	<i>C. affinis</i>	4
29602	Collection locality unknown, Cal. Geol. Survey, collection date unknown	<i>C. affinis</i>	2
45084	Ciudad de Panamá, Panama, J. Zetek, collection date unknown	<i>C. affinis</i>	2
68798	Guaymas, Sonora, Mexico, C. R. Orcutt, collection date unknown	<i>C. bajaensis</i>	1
79238	San Felipe, Baja California, Mexico, J. M. Reed, 12.II.1928	<i>C. bajaensis</i>	3
100077	Ciudad de Panamá, Panama, J. Zetek, collection date unknown	<i>C. affinis</i>	3
110492	La Libertad, Sonora, Mexico, H. N. Lowe. II.1935	<i>C. bajaensis</i>	3
140875	Guaymas, Sonora, Mexico, H. R. Turner, 11.I.1942	<i>C. bajaensis</i>	2
148839	Bahía de San Carlos, Sonora, Mexico, F. Baker & L. G. Hertlein, 1921	<i>C. bajaensis</i>	10
148839	Bahía de San Carlos, Sonora, Mexico, F. Baker & L. G. Hertlein, 1921	<i>C. affinis</i>	2
174438	Puerto Peñasco, Sonora, Mexico, R. C. Beck, 30.IX.1948	<i>C. bajaensis</i>	2
176271	West Coast of Panama, C. M. Dumbauld, collection date unknown	<i>C. affinis</i>	1
198526	San Felipe, Baja California, Mexico, J. E. Fitch, 2.IV.1953	<i>C. bajaensis</i>	2
215636	Guaymas, Sonora, Mexico, C. Field, 1957	<i>C. bajaensis</i>	2
221093	Puertecitos, Baja California, Mexico, E. P. Chace. 11.II.1925	<i>C. bajaensis</i>	1
233102	Sandy Cove, Lagoon, Guayamas, Sonora, Mexico, J. W. R. & A. H. R., II.1940	<i>C. affinis</i>	4
245090	SW side of Bahía de las Ánimas, Gulf of California, Mexico, R. H. Parker, I.IV.1959 [28°55'N, 113°31'W]	<i>C. bajaensis</i>	2
245125	Sargento (Sargent's Point), Sonora, Mexico, W. Emerson, III–IV.1962	<i>C. bajaensis</i>	2
263736	Venado, Panama, T. Dranga, XII. 1938	<i>C. affinis</i>	1
302867_DRY	El Requesón, 17 mi S of Mulegé, Baja California Sur, Mexico, S. P. & H. H. Kool, XII.1992	<i>C. bajaensis</i>	6
302867_WET	El Requesón, 17 mi S of Mulegé, Baja California Sur, Mexico, S. P. & H. H. Kool, XII.1992	<i>C. bajaensis</i>	11
302932	Small Bay, 15 mi S of Mulegé, Baja California Sur, Mexico, S. P. & H. H. Kool, 31.XII.1992	<i>C. affinis</i>	2
339587	San Felipe, Baja California, Mexico, J. Q. Burch, III.1938	<i>C. bajaensis</i>	2
339589	Guaymas, Sonora, Mexico, J.Q. Burch, 1947–1948	<i>C. bajaensis</i>	4
339589	Guaymas, Sonora, Mexico, J. Q. Burch, 1947–1948	<i>C. affinis</i>	11

(continues)

**Table 4.2.** List of specimens examined for morphological comparisons, including MCZ accession numbers, collecting localities, species, and number of specimens (continued).

MCZ Accession No.	Collection Site	Species	No. of Specimens
339592	Guaymas, Sonora, Mexico, J. Q. Burch, I.1948	<i>C. bajaensis</i>	3
339593	Bahía la Choya, Puerto Peñasco, Sonora, Mexico, T. & B. Burch, 25.XII.1966	<i>C. bajaensis</i>	1
339594	Guaymas, Sonora, Mexico, Mrs. H. R. Turver, II.1940	<i>C. bajaensis</i>	1
339595	Mazatlán, Mexico, J. Q. & R. Burch, XII.1960	<i>C. affinis</i>	4
339596	Mazatlán, Sinaloa, Mexico, collector and collection date unknown	<i>C. affinis</i>	4
339605	Carrizal Bay, Colima, Manzanillo, Mexico, L. & C. Shy, 1973–1974	<i>C. affinis</i>	1
339607	San Carlos, Guaymas, Sonora, Mexico, B. L. Burch, 6.I.1963	<i>C. affinis</i>	1
339608	San Carlos, Guaymas, Sonora, Mexico, B. L. Burch, collection date unknown	<i>C. affinis</i>	2
339609	San Carlos, Guaymas, Sonora, Mexico, J. Q. Burch, 3.XIII.1962	<i>C. affinis</i>	1
339610	Bahía la Choya, Puerto Peñasco, Sonora, Mexico, B. L. Burch, I.1967 [31°21'N, 113°41.2'W]	<i>C. affinis</i>	1

sequenced using primer pair 16Sa – 16Sb (Xiong & Kocher, 1991; Edgecombe et al., 2002). ITS-2 was amplified and sequenced using primer pair UCYTB144F and UCYTB272R (Merritt et al., 1998). The cytochrome b (CYT B) gene was amplified and sequenced using primer pair COBF and COBR (Passamonti, 2007).

PCR amplifications (25 µl) were conducted using 1 µL of the template DNA, 1 µL of each [100 µM] primer, 2.5 µL of EconoTaq 10X PCR buffer containing 15 mM MgCl<sub>2</sub> (Lucigen), 0.25µL of dNTP's 100 mM, and 1.25 U of EconoTaq DNA polymerase (Lucigen). The PCRs were carried out using an Eppendorf Mastercycler epgradient thermal cycler. The thermal cycle program consisted of an initial denaturation at 95°C for 2 min, followed by 36 cycles of denaturation step at 95°C (45 s), annealing at 43–48°C (CYT B) or 48–53°C (16S rRNA and ITS 2) (1 min) and elongation at 72°C (90 s). A final elongation step at 72°C (4 min) and a rapid thermal ramp for 4°C were applied to finalize the process.

The double-stranded PCR products were visualized by agarose gel electrophoresis (1.5% agarose), cleaned with 2 µl of diluted (1:3) ExoSAP-IT (USB Corp., Cleveland, Ohio, U.S.A.) in a volume of 22 µL PCR product and performed at 37°C for 30 min followed by enzyme inactivation at 80°C for 15 min. Sequencing reactions were performed in a 10-µL reaction volume using 3.2 µL of primer (1 mM), 1 µL of ABI BigDye™ Terminator v. 3.0 (Applied Biosystems), 0.5 µL of BigDye 5 Sequencing Buffer (Applied Biosystems) and 3.3 µL of cleaned PCR product. The sequencing reaction, performed by using the thermal cycler described above, involved an initial denaturation step for 3 min at 95°C, 25 cycles (95°C for 10 s, 50°C for 5 s and 60°C for 4 min) and a rapid thermal ramp to 48°C. The BigDye-labeled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, Maryland, U.S.A.). The chromatograms were visualized, edited and assembled using Sequencher™ (Gene Codes Corporation, 1991–2011).

Amplification primers were cropped and discarded from the edited sequences.

Multiple sequence alignment of molecular data (Table 4.1) was performed using MAFFT v. 6 using the default strategy (Katoh et al., 2008) for each individual gene region. Haplotype networks were inferred using the statistical parsimony procedure implemented in the program TCS v. 1.21 (Clement et al., 2000), under default settings and assumptions (at 95% confidence interval), with indels treated as a fifth state.

### *Morphometric Analysis*

Three morphological variables were measured following Soares et al. (1998): shell length (anterior-posterior), height (ventrodorsal), and width (left-right) to 0.01 mm with Mitutoyo Absolute™ digital read calipers. Only specimens with complete and intact valves were measured; all single valve specimens were excluded (Table 4.2). Variance ratio tests ( $p = 0.7565$ ), Shapiro-Wilk W test for normal data (W/H,  $p = 0.39339$ ; H/L,  $p = 0.13913$ ), and oneway ANOVA on morphological comparisons between width vs. height (W/H) and height vs. length (H/L) were performed in StataMP 12.0 (StataCorp, 1985–2011).

## **Results**

### *Molecular Analyses*

Specimens collected in two contrasting habitats (sandy and rocky substrata) from two locations (Puerto Peñasco and La Paz) show habitat-specific morphological variation (Figs. 4.1, 4.5–8) and comprise two genetically distinct groups. Based on descriptions by G. B. Sowerby I (1833), specimens from La Paz (byssally attached to rocks) have been identified as *Carditamera affinis*. Morphologically, the specimens found in sandy sediments



**Figure. 4.5–4.8.** Representative morphologies of alcohol-preserved specimens used in the molecular analysis. FIG. 4.5: *Carditamera bajaensis*, sp. nov. (MCZ DNA103800\_1) collected near Puerto Peñasco in exposed sand during low tide; FIGS. 4.6–8: *Carditamera affinis* specimens (MCZ DNA106147\_1-3) collected off the coast of La Paz in and under rocks.

**Table 4.3.** List of *Carditamera bajaensis*, sp. nov. MCZ accession numbers, collecting localities, and number of specimens examined for species description.

MCZ Accession No.	Collection Site	No. of Specimens
79238	San Felipe, Baja California, Mexico, J. M. Reed, 12.II.1928	3
110492	La Libertad, Sonora, Mexico, H. N. Lowe, II.1935	3
140365	Golfo de California, San Juan, Baja California Sur, collector and collection date unknown	31
140875	Guaymas, Sonora, Mexico, H. R. Turner, 11.I.1942	2
148839	San Carlos Bay, Sonora, Mexico, F. Baker & L. G. Hertlein, 1921	12
174438	Puerto Peñasco, Sonora, Mexico, R. C. Beck, 30.IX.1948	2
198526	San Felipe, Baja California, Mexico, J. E. Fitch, 2.IV.1953	2
221093	Puertecitos, Baja California, Mexico, E. P. Chace, 11.II.1925	1
245090	SW side of Las Animas Bay, Gulf of California, Mexico, R. H. Parker, 1.IV.1959 [28°55'N, 113°31'W]	2
245125	Sargento (Sargent's Point), Sonora, Mexico, W.k. Emerson, III-IV.1962	2
254091	SW side of Las Animas Bay, Gulf of California, Mexico, R. H. Parker, 1.IV.1959 [28°55'N, 113°31'W]	2
302867_DRY	El Requeson, 17 mi S of Mulege, Baja California, Mexico, S. P. Kool & H. H., XII.1992	6
302867_WET	El Requeson, 17 mi S of Mulege, Baja California, Mexico, S. P. & H. H. Kool, XII.1992	11
339587	San Felipe, Baja California Norte, Mexico, J. Q. Burch, III.1938	3
339589	Guaymas, Sonora, Mexico, J. Q. Burch, 1947–1948	4
339592	Guaymas, Sonora, Mexico, J. Q. Burch, I.1948	3
339593	Cholla Bay, Puerto Peñasco, Sonora, Mexico, T. & B. L. Burch, 25.XII.1966	1
339594	Guaymas, Sonora, Mexico, Mrs. H. R. Turver, II.1940	2

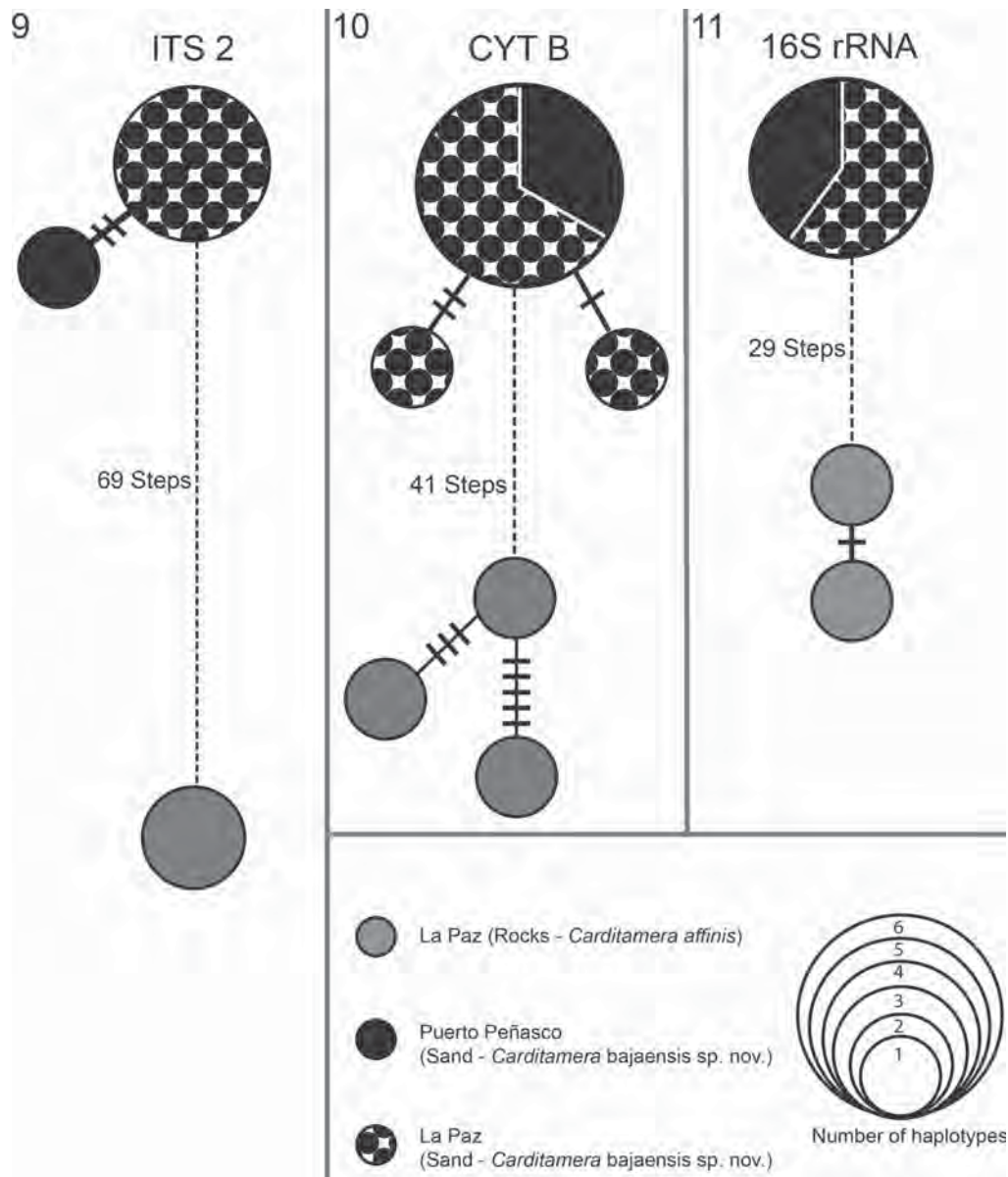


at both locations are narrower and lack spines on the posterior ribs.

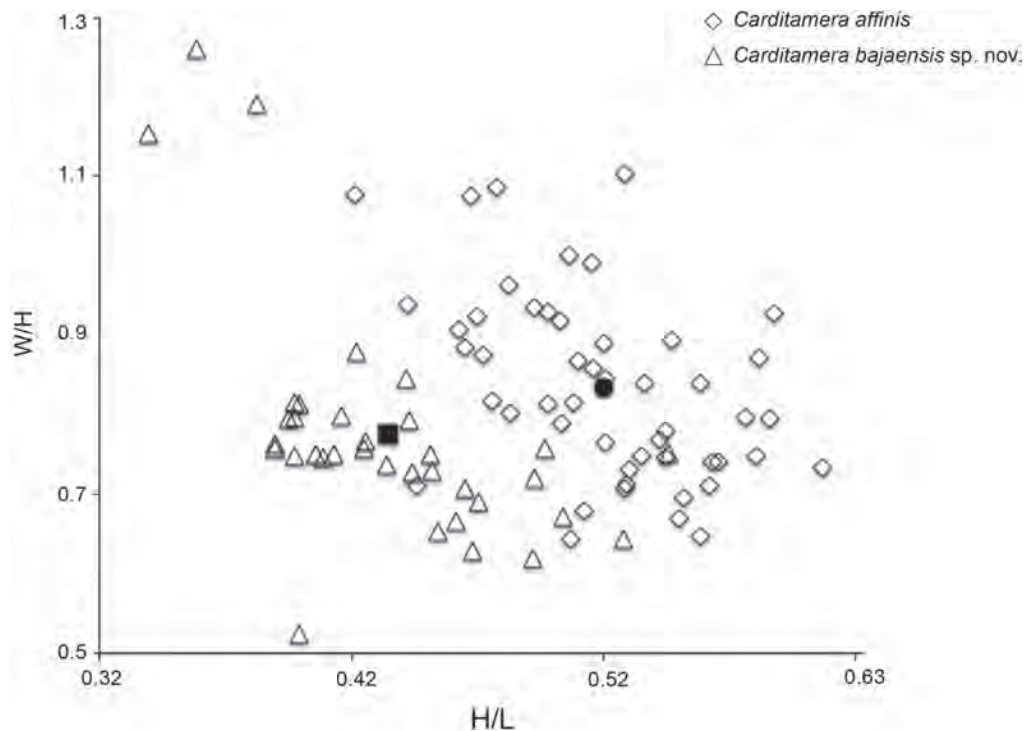
Haplotype analysis shows disconnected networks for all loci (ITS-2, 16S, CYT B) (Figs. 4.9–11). All three gene regions show a clear distinction between the haplotypes collected in the sandy substrata (Puerto Peñasco [sand] + La Paz [sand]) versus haplotypes collected on rocky substrata [La Paz (rocks)]. Small byssally attached individuals have also been observed intertidally in rocks at La Paz, but the specimens were not collected (G. G., March 2003). After relaxation of the 95% confidence interval, 69 mutational steps are required to connect the sandy versus rocky haplotypes, while only two steps separate the sandy substratum haplotypes for the ITS-2 gene region (Fig. 4.9). CYT B and 16S rRNA gene information require 41 and 29 mutational steps to connect the haplotypes, respectively (Figs. 4.10, 4.11). No haplotypes are shared between rocky (*C. affinis*) and sandy (*C. bajaensis*, sp. nov.) forms for any marker.

### *Morphometric Comparisons*

Morphological comparisons between width vs. height (W/H) and height vs. length (H/L) revealed a significant difference in shell shape between *C. affinis* and *C. bajaensis*, sp. nov., which further corroborates morphological distinctiveness (One-way ANOVA,  $p = 0.0419$ ). *Carditamera affinis* specimens were wider and rounder, whereas *C. bajaensis* specimens were thinner and flatter (Fig. 4.12).



**Figure 4.9–11.** TCS networks. FIG. 4.9: Network based on ITS-2 data; FIG. 4.10: Network based on CYT B data; FIG. 4.11: Network based on 16S rRNA data. Representative haplotypes from the three localities are indicated above. The size of the circle is proportional to the number of represented haplotypes. Solid lines connect haplotypes with a single step (inferred intermediate haplotypes are indicated by a hash mark); a dashed line represents relaxation of 95% confidence limit.



**Figure 4.12.** Morphometric comparisons of *C. bajaensis*, sp. nov. (Triangles) and *C. affinis* (Diamonds). Measurements of W/H plotted against H/L; Means of W/H and H/L for *C. bajaensis*, sp. nov. (filled square) and *C. affinis* (filled circle) are 0.78 and 0.43; and 0.84 and 0.51, respectively.

## Systematics

### *Taxonomy*

Carditidae Férussac, 1822

Type genus: *Cardita* Bruguière, 1792

Type species: *C. variegata* Bruguière, 1792, by subsequent designation of Gray, 1847

*Types:* *Carditamera bajaensis*, sp. nov. Figs. 4.1, 4.5, 4.13, 4.14

Additional Material Studied: Eighteen lots, both dry shell collections (n = 17 lots) and one alcohol-preserved lot (MCZ 302867), totaling 92 specimens housed in the Museum of Comparative Zoology, Department of Malacology (Table 4.3). Holotype: (MCZ DNA106146\_1) (41 mm long x 18 mm high x 13 mm wide) from Bahía Balandra (24°19.019'N, 110°19.27'W), La Paz, Baja California Sur, Mexico, depth 1 m, collected 28 February 2009 by V. L. González & G. Y. Kawauchi (on sandy bottom substratum). Paratypes: Six specimens (MCZ DNA 106146\_2-7), same collecting data as holotype; two specimens (MCZ DNA103800\_1-2) (78 mm long x 32 mm high x 26 mm wide) collected from Bahía la Choya (31°20'37"N, 113°38'38"W), Golfo de California, Puerto Peñasco, Sonora, Mexico, depth 1 m, collected 27 March 2003 by G. Giribet (in exposed sand); and two specimens (MCZ 245090), collected from the southwest side of Bahía de las Ánimas (28°55'N, 113°31'W), Golfo de California, Baja California Sur, Mexico, collected 1 April 1959 by R. H. Parker.

Material Examined for Comparison *Carditamera affinis* (G. B. Sowerby I, 1833): three syntypes (NHMUK) collected in the Bahía de Montejó and Golfo de Nicoya, Costa Rica.

*Carditamera cf. affinis* (formerly *Cardita californica* Deshayes, 1854): 3 syntypes (NHMUK) from the collection of Hugh Cuming, collected in the Golfo de California.

### *Etymology*

The specific epithet refers to the state where the type locality of the holotype specimen is found, Baja California Sur, Mexico.

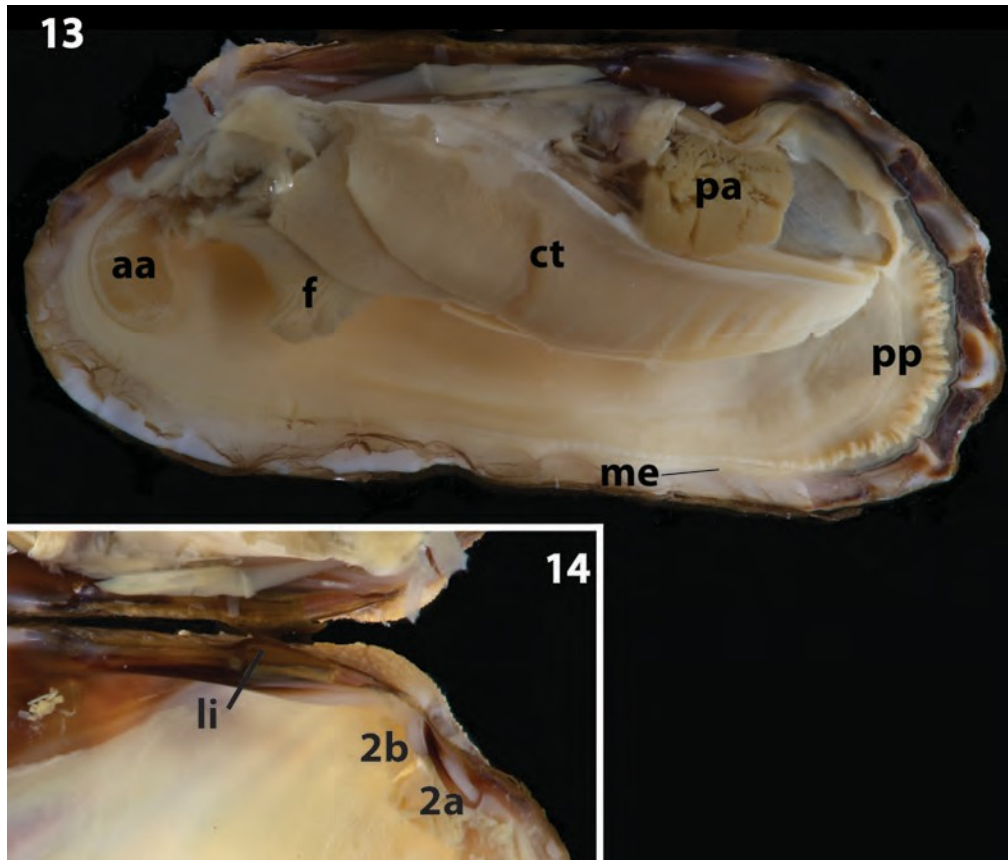
### *Diagnosis*

Moderately sized *Carditamera*, 41 to 85 mm long in measured specimens, narrow, ribbed, thick-shelled bivalve. Shell elongate, quadrangular, twice as long as high, with approximately 15 smooth flat prominent ribs of equal distinctness over the whole shell; longer posteriorly. Color white, banded with brown; lacking spinose posterior ribs like those of *C. affinis*. Pilose thick periostracum; brown coloration.

### *Description*

Posteriorly elongate shell with hinge line parallel to straightened ventral margin (Fig. 4.1). Interior color whitish with brown coloration, non-nacreous. Shell equivalve and inequilateral. Umbones anterior, prosogyrate. Lunule small and deep; escutcheon weakly distinct. Shell margin crenulate, both in the interior and exterior of the valves, following the radial ribs. Pilose periostracum only apparent around margins (Fig. 4.13).

Hinge plate wide. Hinge structure with strong lateral teeth, 2 right anterior laterals (la1 and la2) and one right anterior cardinal (3a) and two corresponding left anterior cardinal teeth (2a and 2b) (Fig. 4.14). External ligament present, opisthodontic, parivincular. Pallial line complete, without pallial sinus. Papillate posterior mantle margin present, with the posterior mantle folds fused to form posterior excurrent aperture; mantle margins not fused ventrally. Carinate foot pointed posteriorly. Byssal groove present and with byssus occasionally present in adult stage [2 of N = 8], yet rare. Anterior and posterior pedal retractors muscles present.



**Figure 4.13 & 4.14.** Alcohol-preserved specimen of *Carditamera bajaensis*, sp. nov. (MCZ DNA106146\_1). FIG. 4.13: Animal with left mantle and valve removed; FIG. 4.14: Left valve showing hinge and cardinal teeth. Abbreviations: aa, anterior adductor muscle; me, mantle edge; ct, ctenidium; f, foot; li, ligament; pa, posterior adductor muscle; pp, posterior papillae; 2a, left anterior cardinal tooth; 2b, left middle cardinal tooth.



**Figure 4.15.** Map of the Golfo de California showing known localities for *Carditamera bajaensis*, sp. nov. Exact collecting localities represented by a black square; approximate collecting localities indicated by a black triangle based on Table 4.3.

Heteromyarian, with a slightly larger posterior adductor; the smaller anterior adductor attached to an antero-ventral extension of the valves. Eulamellibranch ctenidia, homorhabdic, with outer demibranch slightly smaller than inner demibranch. Labial palps small, triangular in shape. Stomach structure not elucidated, with a spiral typhlosole. Midgut uncoiled.

### *Habitat*

Semi-infaunal; specimens have been collected buried in exposed and intertidal sandy beach environments.

### *Distribution*

Known from several collecting localities within the Golfo de California (Fig. 4.15).

## **Discussion**

The three syntypes of *Carditamera affinis* (NHMUK) (one specimen pictured in Fig. 4.2) are large elongate shells, in which the length is about twice the height, and have 15 or more ribs present. Prominent posterior scales are present on the exterior of the shell and specimens have coloration ranging from brownish white to brown. *Carditamera affinis* is more globose than *C. bajaensis*, and the shell is slightly broader than deep. Compared with *C. affinis*, *C. bajaensis* is more elongate, less globose, has a more linear ventral margin and lacks prominent large posterior scaly projections. *Carditamera affinis* is epifaunal, found in crevices or under rocks, usually byssally attached to the substratum, sometimes exposed in the intertidal zone; *Carditamera*



*bajaensis* is semi-infaunal and has been collected in exposed sandy beach environments at low tide. To evaluate the validity of *C. bajaensis*, all five known synonyms of *C. affinis* – *Cardita californica* Deshayes, 1854; *Cardita incerta* Clessin, 1888; *Cardita petunculus* Reeve, 1843; *Cardita picta* Clessin, 1888; *Cardita volucris* Reeve, 1843 – were investigated and each original description was checked.

As indicated by Keen (1971: 107), *Carditamera* cf. *affinis* (formerly *Cardita californica* Deshayes, 1854) was described as a smoother northern subspecies of *Carditamera affinis* (one specimen pictured in Fig. 4.3). However, upon reexamination of the syntypes of *Cardita californica* (NHMUK) (Fig. 4.3), the specimens have globose elongate shells with distinct posterior scales, and are similar in shape and periostracum color to those of the *C. affinis* syntypes, unlike the smooth exterior and narrower width of *C. bajaensis*, further indicating that *Cardita californica* and *C. affinis* are conspecific.

Clessin (1888) described two species that have subsequently been synonymized with *C. affinis*. Both *Cardita picta* Clessin, 1888, and *Cardita incerta* Clessin, 1888, were described as having wide rounded shells with a total of 19 radial ribs, differing from the narrow shell and 15 ribs of *C. bajaensis*. Reeve (1843) also described two species that have been synonymized with *Carditamera affinis*: *Cardita petunculus* Reeve, 1843, is a large form from Madagascar that lacks banding and spotted coloration characteristic of *C. bajaensis*, and *Cardita volucris* Reeve, 1843 (type locality unknown), which has an elongate, globose, and scaly shell, and appears to be a true synonymy of *C. affinis*.

Haplotypic networks and phylogenetic analyses have been recently used to elucidate cryptic species of marine invertebrates (e.g., Baker et al., 2007; Duran & Rützel, 2006; Kawauchi & Giribet, 2010), including molluscs (e.g., Marko & Moran, 2009; Kawauchi &

Giribet, 2011; Zamborsky & Nishiguchi, 2011), as they have the power to compare the degree of within species and among species genetic diversity. The case of *Carditamera bajaensis* is clear, as all characters, from shell anatomy (little data are available for the internal anatomy of related *Carditamera* spp.), ecological niche, and haplotypes, support the reciprocal monophyly of the sampled populations. Furthermore, the specimens here described as *Carditamera bajaensis* cannot be assigned to any of the previously described species now considered synonyms of *Carditamera affinis* or any other *Carditamera* described from this region. This, in addition to the molecular sequence data that distinguish the two life forms for every examined marker, but that unite populations of the infaunal species separated by about 800 km, confirms a distinct and new species, *Carditamera bajaensis*. Ongoing revision of Carditidae, in concert with molecular tools, is anticipated to contribute significantly to the systematics and known diversity of constituent genera.

Evidence for cryptic speciation in the light of phenotypic plasticity has been investigated within several molluscan groups (Richter et al., 2008; Kawauchi & Giribet, 2011). Traditionally, taxonomy within Bivalvia has relied heavily on characters of shell morphology. However, reliance on shell morphological characters alone, which has a propensity in conchiferans for both cryptic speciation (e.g., Won et al., 2003b; Lee & Ó Foighil, 2004; Johnson et al., 2009; Lorion et al., 2010) and environmental plasticity (Yeap et al., 2001; Wulschleger & Jokela, 2002; Baker et al., 2003, 2004; Hollander et al., 2006; Pfenninger et al., 2006; Lorion et al., 2010), can belie the estimation of diversity within these groups.

Convergence and parallelism are rampant within Bivalvia, not only with respect to morphology, but also mode of life (Alejandrino et al., 2011), and more integrated approaches are necessary to delimit species (Harper et al., 2000). This approach is exemplified

by the study of Ritcher et al. (2008), in which morphological, in concert with genetic and ecological data, were used to describe a distinct new species of *Tridacna* in the Red Sea; previously, large phenotypic plasticity had mistakenly been ascribed to a single taxon.

Evolutionary plasticity of life habitat has played an important role in bivalves, both in response to environmental changes (Soares et al., 1998) and for driving diversification (Marko & Jackson, 2001). Environmental plasticity may have facilitated colonization of new habitats by *Carditamera*, followed by niche partitioning and ecological speciation (e.g., in habitats with varying substrata). The sympatric distributions of *C. affinis* and *C. bajaensis*, and the segregation of their respective substrate type is suggestive of a role for evolutionary plasticity as a driver for the diversification within the genus in the Golfo de California. Testing this hypothesis utilizing morphological, ecological, and population genetic data requires denser sampling of *Carditamera* than is currently available, and therefore remains an objective for future investigations.

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